University of Alberta

Molecular Genetics and Characterization of Low Temperature Responses in *Brassica napus*.

By

Glen Patrick Hawkins ©

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Research in partial fulfillment of the requirements
For the degree of Doctor of Philosophy
in
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Department of Plant Science

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ABSTRACT

Exposure to low-temperature induces a number of simultaneous phenotypic responses in some plants that enable them to overwinter, and flower successfully in the spring. These include freezing tolerance, adjustment to low temperature, and vernalization. A major objective of this study was to design a plant system able to separate the individual responses induced by low-temperature. Microspore technology was used to develop a population of double haploid plants (DH) from *Brassica napus* by crossing freezing tolerant cv. Cascade (TL50 = -15.5°C) with freezing sensitive cv. Rebel (TL50 = -7.5°C). Within the resulting material, acclimation-specific freezing tolerance was genetically separated from inherent freezing tolerance and vernalization.

The population displayed transgressive segregation for freezing tolerance, with acclimation-specific tolerance ranging from –3°C to greater than –18°C and inherent tolerance ranging from –2°C to –8.5°C. These results indicate a potential for increased freezing tolerance in winter canola. In addition, the ability to genetically separate these traits provides an opportunity to study them on an individual basis.

Several DH lines were analyzed to determine whether certain low-temperature induced genes segregate with freezing tolerance. As they did not, this raised the possibility that differences in freezing tolerance may be reflected in differences in gene copy number or expression. The genetic relationship of BN28, a low-temperature induced gene, was studied in the *Brassicaceae*. The results showed that unique BN28 homologues are present in all the diploid progenitors, and are not inherited equally in all the allotetraploids represented in the *Brassicaceae*. All species show message induction but some fail to accumulate protein, indicating translational regulation.

Further analysis of the population identified one line, VERN-, that had lost the need for vernalization and behaved as a spring type. Despite the loss of a vernalization requirement, VERN- retained a very high degree of both inherent and acclimation-specific freezing tolerance. The VERN- line is currently being assessed for possible cultivar registration. Genetic analysis of VERN- revealed a distinct polymorphism near a linkage group previously reported by Ferriera et al. (1995). Results indicate that the linkage between freezing tolerance and vernalization can be separated and analyzed independently in winter canola.

Abbreviations

ANOVA. Analysis of variance
BC$_1$. Backcross first generation
BSA. Bovine serum albumin
cas. Cascade
cDNA. Complementary Deoxyribonucleic acid
cv. Cultivar
DEPC. Diethyl pyrocarbonate
ddH$_2$O. Double distilled deionized water.
DNA. Deoxyribonucleic acid
dNTP. Deoxyribonucleotides
DH$_1$, DH1. Double haploid – first generation
EDTA. Disodium ethylenediaminetetra-acetate
F$_1$, F1. First generation offspring
F$_2$. Second generation offspring
FT/ft. Freezing tolerance
GLM. General linear model
Kd. Kilodalton
Kb. Kilobase
LT$_{50}$. Temperature at which 50% death occurs
mRNA. Messenger Ribonucleic acid
MW. Molecular weight
PCR. Polymerase chain reaction
RAPD. Randomly amplified polymorphic DNA
reb. Rebel
RFLP. Restriction fragment length polymorphism
RNA. Ribonucleic acid
rpm. Revolutions per minute
SAS. Statistical analysis software
SDS. Sodium dodecylsulfate

SDS-(PAGE) Sodium dodecylsulfate-polyacrylamide gel electrophoresis

S.E. Standard error

SSC. (salt, sodium citrate) buffer

TAE. 10mM Tris, 1% acetic acid, 1mM EDTA

TBS. Tris buffered saline

TE. 10mM Tris, 1mM EDTA

TL_{50}. Temperature at which 50% ion leakage occurs

Tris. Tris(hydroxymethyl) methylamine

QTL. Quantitative trait loci

VERN-. No vernalization requirement

VERN+. Requiring vernalization

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CHAPTER 1

Literature Review

1.1. INTRODUCTION

Members of the *Brassicaceae,* including *Brassica napus and Brassica rapa*, are important worldwide as oilseed crops. In registered varieties with low erucic acid and low glucosinolates, the oil found in mature seed is known as canola oil. Increased demand for canola oil has hastened the need for an understanding of the genetic control of agronomic traits (Ferreira et al., 1994). Development of cultivars better suited to the often harsh conditions in the temperate growing regions, such as drought, heat, disease and low temperature, has become a focus for canola breeders.

Progress has been slow in improving tolerance to low temperature and frost. This appears to result from a lack of suitable germplasm, an inability to rapidly and accurately assess tolerance and the complexity of plant responses to low temperature.

With the advent of microspore culture, the ability to rapidly produce stable populations for study has become a reality. This approach, coupled with ion leakage as an accurate measure of freezing tolerance, has created the potential to study the relationship between responses to low temperature in the

Brassicaceae, including inherent freezing tolerance, acclimation-specific freezing tolerance and vernalization.

1.1.1. Status of Canola in Canada

The production of edible oil seed in the Canadian marketplace has been increasing at a steady pace over the past decade. In 1986, 6.4 million acres was planted in canola. Production peaked in 1994, when over 14 million acres was seeded. This has since declined to approximately 8.6 million acres in 1996 Canola Council, 1996). All of the major canola cultivars presently being produced in Canada are from the *B. napus* (Argentine) and *B. rapa* (Polish) species. The term canola is a registered trademark of the Canola Council of Canada and is used to describe edible rapeseed cultivars. Canola must meet standards based on seed, meal and oil characteristics. The seed must be of the genus *Brassica* and contain <2.0% of all fatty acids as erucic acid (22:1) and <30 µmoles of total glucosinolates per gram of defatted meal at a moisture content of 8.5%. (Canola Growers Manual, 1991). Proposed changes to the definition of canola will eventually see these levels reduced to <1.0% erucic acid and <18 µmoles of total glucosinolates (Canola Council of Canada, 1997).

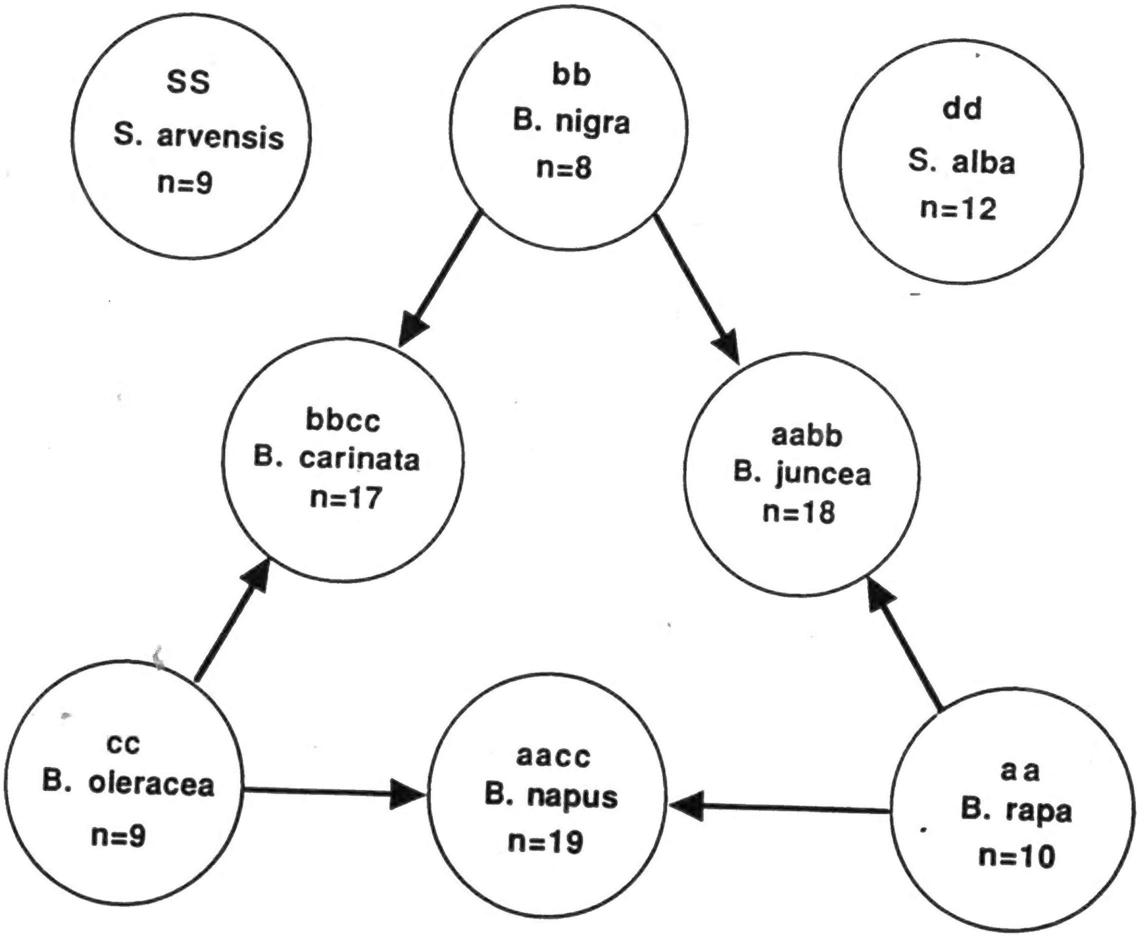
1.2. Origin of Oilseed Brassicas

History shows that rapeseed was cultivated as early as the 20th century B.C. in India and then later moved to China and Japan (Canola Growers Manual, 1991). Rapeseed has the ability to grow in the temperate regions of the world. This eventually led to the production of canola quality rapeseed in temperate regions such as Canada and Northern Europe. Both winter and spring varieties of canola are available, but winter cultivars are preferred in parts of Europe and Asia because of their increased yield potential over spring cultivars. Production in Canada has seen limited success because of a lack of adequate levels of freezing tolerance (Canola Growers Manual, 1991).

The ancestry of the *Brassicaceae* was first described by Morinaga (1934), who showed that three diploid progenitor species, *Brassica nigra* (BB, n=8), *Brassica oleracea* (AA, n=10), and *Brassica rapa* (CC, n=9) (originally *Brassica campestris*) gave rise to three allotetraploid species, *Brassica carinata* (BBCC, n=17), *Brassica juncea* (AABB, n=18) and *Brassica napus* (AACC, n=19). This relationship was confirmed by U (1935) who succeeded in artificially combining *B. rapa* (CC) and *B. oleracea* (AA) to form the allotetraploid *Brassica napus* (AACC). The relationship between species as outlined, is now accepted as U's triangle (1935) (Fig 1.1). Subsequent allotetraploids have been produced according to this triangle relationship using the original progenitor species

Brassica oleracea, *Brassica rapa*, and *Brassica nigra* (Downey et al., 1975; Olson and Ellerstrom, 1980).

Figure 1.1. U's Triangle. Genetic relationship within the *Brassicaceae.*



* *Brassica campestris* is now known as *Brassica rapa*.

1.3. Effects of Low Temperature on Plants

Levitt (1980) defined biological stress as any change in environmental conditions that might reduce or adversely affect growth or development. Environmental stress can be divided into biotic stress and physiochemical stress. Biotic stress is imposed by infection or competition of other organisms. Physiochemical stress is imposed by extremes in conditions such as temperature, water potential, chemical effects, wind and exposure to radiation. In this study, I will confine the scope of stress to that incurred by exposure to low temperature. In freezing tolerant plants, exposure to low temperature triggers a series of responses including low temperature stress, low temperature adjustment, the acquisition of acclimation-specific freezing tolerance (acclimation), and vernalization.

Adjustment to low temperature stress and acclimation occur simultaneously and are, therefore, hard to differentiate. To combat the effects of low temperature, plants must undergo adjustment. During the adjustment period, accumulation of metabolites has been documented (Levitt, 1980). Similarly, acclimation is the result of a myriad of biochemical and physiological processes (Guy, 1990). Differential expression of mRNAs and proteins has been reported to occur during the acclimation process (Guy and Haskell,1987; Gilmour et al., 1992; Boothe et al.,1995). Accumulation of carbohydrates and changes in membrane lipid composition have also been documented (Thomashow, 1990).

What is not known, however, is whether the changes lead to adjustment and growth at low temperature or the attainment of freezing tolerance, or both. This creates a need for a better understanding of the processes that occur during exposure to low temperature and requires a biological system that can separate the individual components that are triggered by low temperature.

Exposure to low temperature not only induces freezing tolerance and adjustment to low temperature, but is also required for vernalization in winter species. Fulfillment of vernalization requirements results in the transition from vegetative to reproductive growth (Dennis et al., 1996).

Little is known about the genetics of vernalization and how it is related to freezing tolerance. Previous work in winter wheat suggests that vernalization and freezing tolerance share an obligate relationship (Fowler et al,. 1996). Concurrent with my work, Galiba et al. (1995) showed that recombination between specific freezing tolerance and vernalization loci was possible in winter wheat. Mapping studies in *B. rapa* have revealed that four linkage group QTLs (quantitative trait loci) appear to have a role in freezing tolerance (Teutonico et al., 1995) There is also two linkage group QTLs which independently segregate for vernalization (Ferreira et al., 1995). The apparent lack of genetic linkage between the two phenotypic traits of freezing tolerance and vernalization requirement suggests that they will eventually be separated phenotypically as well. This will be addressed in the present study.

1.4. Breeding Strategies in Brassica

Production of new cultivars has generally made use of traditional methods of plant breeding, coupled with phenotypic and genotypic assessment. Genotypic selection is based on selection of the genes inherited by an individual from its parents. Phenotypic selection is based on the manner in which genes are expressed and observed as differences in physiology or morphology (Suzuki et al., 1988). Except when the trait is under strong genetic control, such as single gene traits or high heritability, phenotypic selection can be difficult to interpret as phenotypes can be subject to environmental effects. In plant breeding, a phenotype can be measured using qualitative traits such as plant vigour and general appearance, or quantifiable traits such as yield, oil and protein composition (Thurling, 1993). Improving a particular cultivar requires a combination of both art and science. The art of breeding is the ability to visually select potential parent lines holding traits of interest, and the science is the ability to genetically combine and select the desired phenotype in the offspring. In complex systems, such as low temperature responses, this selection process is difficult.

1.4.1. Pedigree Breeding

The pedigree method of breeding is used in populations of self- and cross- pollinated species for the development of desirable homogeneous lines (Fehr,

1993). Pedigree breeding produces F_1s by hybridization of two parental lines, which are then grown to maturity for F_2 seed (Snape, 1982). Thus, F_2 individuals are recombinant products of the original parents that will continue to segregate in further generations until fixation occurs at approximately F_6. To stabilize the genotype, successive rounds of self-pollination and selection are required. Generally, sufficient homozygosity can be attained after five or six self-pollinated generations (Fehr, 1993). The time needed to complete the selfing process is a major limitation of this approach, especially where environmental interactions require seasonal field assessment and selection of individual generations. On the other hand, an advantage of the pedigree method is that recombination occurs during each meiotic generation allowing for expression and selection of new combinations of favorable traits.

1.4.2. Doubled Haploid Breeding

Doubled haploid (DH) breeding makes use of the ability to regenerate individuals from gametes, without the need for fertilization. To be successful, tissue culture technology must be able to produce large numbers of embryos representing the genetic diversity of the parents. An advantage of this system is that whole plants are regenerated from individual cells, each having a unique genotype.

Haploid embryo production in the *Brassicaceae* is achieved by the isolation of microspores from the pollen sac. At this stage, the microspore is uninucleate and has not yet undergone the first mitotic division (Fan et al., 1988). *In vitro* culture of microspores under sterile conditions leads to the formation of haploid embryos. These embryos undergo cell division and grow in a manner similar to zygotic embryos (Wilen, 1992). When placed on appropriate media, (Lichter, 1982) embryos will form roots and shoots not unlike a germinating seed. Unless spontaneous doubling occurs, the resulting DH, plants remain sterile and must have their chromosome number doubled using colchicine. Colchicine is an alkaloid drug that inhibits spindle fiber formation during metaphase and anaphase of mitosis (Suzuki et al., 1988). This prevents separation of the paired chromatids after the centromere splits. The result is a single cell that is homozygous at all loci.

The use of doubled haploid technology in Brassica breeding has increased in recent years and is now being used routinely in many breeding programs (Thiagaragah and Stringam, 1993). To be useful in a breeding program, doubled haploid production must have advantages over conventional pedigree breeding (Cloutier et al., 1995). One advantage of double haploid technology is that it fixes recombinant gametes directly as homozygous lines in a single generation (Snape 1982), whereas five or six generations of self-pollination and selection are needed in conventional pedigree programs. A disadvantage of doubled haploid breeding is the inability to combine favorable loci through generations of recombination (Fehr, 1993). Thiagaragah and Stringam (1993) showed, however, that doubled haploid technology compares favorably with conventional breeding practices.

1.5. Molecular Markers and Mapping

Variations in expressed proteins, termed isozymes, were some of the first molecular markers used for marker assisted selection (Stuber, 1992). With the advent of techniques capable of exposing genetic differences, research has moved towards the use of molecular markers in screening traits of interest. Through the use molecular markers, breeders are bridging the gap between phenotype and genotype (King, 1990). The goal is to use molecular markers to screen and select breeding material for desirable traits. Molecular makers hold certain advantages over phenotypic markers. Molecular markers are phenotypically neutral and are not affected by environmental interactions. They also behave in a codominant manner (excluding RAPDs) distinguishing all genotypic interactions, and are free of epistatic interactions (Stuber, 1992). A disadvantage of molecular markers is they are not usually the gene for the desired trait (Weeden, 1993), but are in close linkage with the trait of interest. The costs and requirements for radioactivity associated with several of the molecular techniques can often dictate which method(s) are used.

The commonly used methods of RFLP and RAPD have allowed us to increase our knowledge of gene location and genome organization (Gillet, 1991). As a result of this, we have a better understanding of how agronomic traits are expressed, related, and inherited. Ideally, molecular markers ensure that desirable traits are recovered and selected, even if they remain unexpressed in terms of phenotype (Edwards et al., 1992), however, molecular marker technology is still in its infancy and requires additional study before it becomes a preferred method of trait selection.

1.5.1. Restriction Fragment Length Polymorphism (RFLP)

RFLPs are genomic markers used to detect differences associated with nucleotide substitution, insertion, deletion, or rearrangement of genomic DNA sequences (Chang and Meyerowitz, 1991). An advantage of this technique and other molecular markers is they permit early screening of traits without the influence of environmental effects (Lander and Bolstein, 1989), whereas phenotypic trait selection can be influenced by effects of the environment. RFLP makes use of the ability of restriction endonucleases to cut genomic DNA into discrete size fragments based on nucleotide sequence. These fragments are then separated according to size using electrophoresis and blotted to a filter (Southern, 1975). The blot is then hybridized with a radiolabeled probe which binds to those restriction fragments having a sufficient degree of homology (Gillet, 1991). The probed blot provides a genetic fingerprint of the plant being studied. It may be possible to then link the fingerprint with the phenotype of interest. Of course, linkage can only be determined if suitable material is studied. This means that careful phenotyping, selection and fixing of genetic traits must precede any RFLP analysis. An advantage of RFLPs is that the codominant nature of the marker can be determined. A phenotypic, or morphological marker has alleles that are inherited in a dominant/recessive manner. RFLPs behave in a codominant manner, whereas RAPDs are only dominant in nature. This allows for the determination of genotype in a plant derived from any two parents

RFLP maps have recently been published for *Brassica napus* (Landry et al., 1991; Lydiate et al., 1993; Ferreira et al., 1995*), Brassica oleracea* (Quiros et al., 1987; Slocum et al., 1990; Landry et al., 1992), and *Brassica rapa* (Song et al., 1991; Chyi et al., 1992; Teutonico and Osborn, 1994). Markers from these maps have been used within the *Brassicaceae*, in identification of the gene(s) controlling traits, including; linolenic acid (Hu et al., 1995), flowering time in *B. oleracea* (Camargo and Osborn, 1996), and vernalization requirement in *B. rapa* (Teutonico and Osborn, 1995). Mapping of traits is presently expanding to include quantitative associations (Teutonico and Osborn, 1994). More work is needed however, before complex traits (QTLs) such as freezing tolerance can be mapped using linked molecular markers.

1.5.2. Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is another molecular technique capable of detecting genomic differences. This molecualr technique was simultaneously described by two independent research groups; Williams et al. (1990) and Welsh and McClelland (1990). Detection of RAPDs involves PCR amplification of DNA segments using an arbitrary primer (Innis et al., 1990).

Products are then separated by gel electrophoresis and visualized by ethidium bromide staining (Rafalski et al., 1991). Specified amplified products segregate in Mendelian fashion and can, therefore, be used as genetic markers (Williams et al., 1990). RAPDs are sensitive enough to detect single base changes between DNA samples (Williams et al., 1990). The use of arbitrary primers means that prior knowledge of the genetic composition of the plant is unnecessary. One drawback to the use of RAPDs is that they yield only dominant markers. Thus, markers can only be scored as present or absent and no inferences as to genotypic modifications causing the polymorphic difference can be made. Again, the plant material must be genetically stable and the phenotype must be defined, if mapping analysis with markers is to be successful.

1.6. Regulation of Freezing Tolerance

Freezing causes the formation of both intracellular and extracellular ice. Intracellular ice formation is rare in plant cells but is considered lethal (Guy, 1990). Ice formation occurs in the extracellular spaces and results in cell dehydration (Levitt, 1980). Extracellular ice preferentially forms as a result of a lower solute concentration of the extracellular fluid as compared to the cytoplasm (Palta and Simon, 1993). As ice crystals continue to grow, intracellular water is osmotically removed in order to maintain equilibrium. The result is freeze-induced dehydration of the cell.

The ability of some plants to acclimate and aquire freezing tolerance involves biochemical and physiological alterations. These include changes in soluble carbohydrates (Krause et al., 1982; Koster and Lynch, 1992; Hurry et al., 1995), altered membrane structure, composition and behaviour (Hincha et al., 1990; Steponkus et al., 1990) and altered physiology including photosynthesis and carbon cycling (Hodgins et al, 1989).

At the level of gene expression, changes in the expression of proteins, amino acids and RNA have been observed (Siminovitch et al., 1968). Recently, a number of low temperature induced genes have been isolated and potential function examined (Cativelli and Bartels, 1990; Guy, 1990; Thomashow, 1990; Orr et al., 1992; Weretilynk et al., 1993; Palva, 1994). Study of these genes has revealed regulation at the level of RNA transcription and translation (Guy, 1990; Boothe et al., 1995;; Hawkins et al., 1996). The absolute role that the low temperature induced genes have in freezing tolerance is still unclear.

Freezing tolerance can be influenced in a number of ways. Cloutier and Siminovitch (1982) showed that freezing tolerance in Rye epicotyls increased following a desication treatment. This was expected to be mediated by abscisic acid (ABA). ABA alone has been shown to increase freezing tolerance in non-acclimated tissues, including suspension culture (Orr et al., 1990). These results suggest that the low temperature induced genes are responsive to dessication and ABA. Many of the cor (cold regulated) genes (cor 6.6, 47 and 115) and rab (regulated by ABA) genes including rab 21 can be regulated by ABA and low temperature, while other genes, including BN28 are only responsive to low temperature (Boothe et al., 1995). This may indicate that BN28 may have a unique role in the attainment of freezing tolerance in plants.

1.7. Breeding Material

The present study made use of DH_1 lines derived from reciprocal crosses between two winter cultivars of *Brassica napus*, cv. Cascade and cv. Rebel. The main reason for choosing these cultivars was their difference in freezing tolerance (TL_{50} Cascade = -15.5°C and TL_{50} Rebel = -7.5°C). Both cultivars are canola quality and were developed at the Agricultural Experimental Station in Moscow, Idaho 83843 by Auld et al. (1987). Cascade was selected in the F6 generation from crosses between Indore and three edible oil lines; Sipal, WW 827, and Liraglu. Segregating generations were advanced by single seed descent and F3 - F6 generations were screened for low levels of glucosinolate in mature seed (<8% seed moisture) (Auld et al., 1987). Rebel is an F6 line derived by a cross between OAC Triton and WRE 17. OAC Triton is a triazine-tolerant spring type from the University of Guelph (Beversdorf et al., 1984). WRE 17 is a line derived by single seed descent from a cross between Sipal and

Indore. Rebel was selected using the same methods that were used for Cascade.

1.8. Objectives

The overall objective of this study was to design and characterize a system to examine the inheritance and segregation of low temperature responses in *Brassica napus*. It was hoped that maximum segregation of freezing tolerance would result from crosses between two winter cultivars having a large difference in freezing tolerance. The integration of doubled haploid technology into the scheme was also expected to contribute to segregation within the population, by permitting expression of recessive traits. Finally, the use of two related cultivars in the initial crosses was anticipated to maximize the combinations of genes for freezing tolerance, and minimize non-related effects from each of the parents.

I hypothesized that low temperature-induced genes would segregate with freezing tolerance in the resulting population. When they did not, I examined the possibility that subtle genetic differences exist in the expression of low temperature-induced genes. This was examined in a number of the *Brassicaceae*, using BN28 as the gene of interest.

The fact that Rebel has a weak vernalization requirement whereas Cascade has a strong vernalization requirement was also exploited. This was expected to result in variation in the vernalization requirement in the DH population. The objective of this portion of the study was to determine the relationship between freezing tolerance and vernalization in *Brassica napus*.

1.9. Literature cited

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CHAPTER 2

Segregation of Freezing Tolerance in Micropspore-Derived DH Lines of *Brassica napus*

2.1. INTRODUCTION

A number of mechanisms have evolved that enable plants to cope with a variety of adverse environmental conditions (Levitt, 1980; Lang et al., 1981; Key and Kosuge, 1984). In order to survive exposure to freezing temperatures, plants must possess high levels of both inherent and acclimation-specific tolerance. Inherent tolerance is genetically determined and allows the plant to cope with short periods of high, sub-zero temperatures. Acclimation, by prolonged exposure to low temperature, increases the degree of freezing tolerance in those plants capable of acclimating. This involves not only complex physiological, molecular and biochemical processes (Singh and Laroche, 1988), but also genetic controls (Stone et al., 1993; Teutonico et al., 1995)

Low temperature acclimation has been shown to cause alterations at all levels of gene expression (Thomashow 1990). Recently, the genes encoding a number of low temperature-induced proteins have been isolated from several plants including *Brassica napus* (Orr et al., 1992; Weretilnyk et al., 1993), *Arabidopsis thaliana* (Kurkela and Franck, 1990; Gilmour et al., 1992; Lin and Thomashow, 1992; Palva, 1994), *Spinacia oleracea* (Guy, 1990), *Hordeum* *vulgare* (Cativelli and Bartels, 1990) and *Medicago sativa* (Monroy et al., 1993). In an attempt to assign function, these genes have been analyzed for regulation of expression, and their products studied with regard to structure and cellular localization (Houde et al., 1992; Lin and Thomashow, 1992; Yamaguchi-Shinozaki and Shinozaki, 1994; Boothe et al., 1995; 1997). Similar studies have revealed that subsets, or families of proteins are regulated at the level of RNA transcription or protein translation during exposure to low temperature (Guy, 1990; Johnson-Flanagan et al., 1991; Hawkins et al., 1996). To date, however, no function has been assigned to any of the low temperature-induced genes described in the literature. This lack of clear function creates a need for a greater understanding of the processes that occur when a plant is exposed to low temperature and demonstrates that there are many responses to low temperature that are not directly related to freezing tolerance.

Selection for freezing tolerant material has been difficult because of the numerous responses associated with exposure to low temperature, including low temperature adjustment and vernalization requirements. Furthermore, many breeders working to improve freezing tolerance have used field survival as the major selection criterion (Marshall, 1982). This is difficult to assess as there are many other factors controlling field survival, including ice encasement and consequent anoxia (Andrews and Morrison, 1992). Selection is further complicated by the fact that the acclimation process is cumulative and can be stopped, reversed and restarted (Gusta et al., 1982; Roberts, 1979). The fact that material must be acclimated for up to 6 weeks for maximum attainment of freezing tolerance has only complicated the breeding and selection process for hardier canola cultivars.

Research on the mode of inheritance of freezing tolerance has led researchers to the conclusion that this trait is complex and follows a polygenic inheritance pattern (Stushnoff et al., 1984). For example, Sutka (1981) showed that 15 of 21 chromosomes can be implicated in freezing tolerance in *Triticum aestivum* (winter wheat). In *Brassica rapa* there appears to a minimum of 4 linkage groups associated with freezing tolerance (Teutonico et al., 1995).

If the percentage of land planted to winter cultivars is going to increase, further increases in freezing tolerance are required. It is difficult to ascertain the highest level of inherent and acclimation-specific freezing tolerance available in winter canola as many breeding programs do not assess freezing tolerance. Nonetheless, one could surmise that the highest tolerances are in the range of –5°C and –15°C for inherent and acclimation-specific freezing tolerance, respectively. This is based on the fact that the most tolerant canola quality material was produced in the US Pacific Northwest by Dick Auld (personal communication). At the beginning of this study, he provided Cascade as a source of freezing tolerant material available to us. Also, previous work showed that both Cascade and Rebel were amenable to tissue culture (unpublished).

The objective of the present study was to design a system to address the complex nature of freezing tolerance in winter *Brassica napus*. Freezing tolerance was assessed in a segregating doubled haploid population (DH) produced using microspore culture. Freeze testing was conducted on up to five self-pollinated generations to ensure that further segregation was not occuring within lines. It was hoped that maximum levels of freezing tolerance would be derived using a winter by winter cross. The relationship between inherent and acclimation-specific freezing tolerance was assessed using phenotypic characterization. Genotypic characterization was used to examine the distribution of acclimation-specific freezing tolerance in the population. The ability to genetically separate the levels of freezing tolerance may provide valuable insight into the individual control of these traits in the *Brassicaceae*.

2.2. MATERIALS AND METHODS

2.2.1. Parent Material and Crossing

Seed of winter type parent cultivars of *Brassica napus* Cascade and Rebel were sown into 15 cm pots containing a soil mixture of peat:vermiculite:sand (2:2:1). Pots were placed in a greenhouse under a 16/8 day/night photoperiod and a minimum light intensity of 300 μmole $m^{-2}s^{-1}$ maintained using high intensity sodium vapor lights when needed (HID Sylvania, Canada). Temperature was maintained at 20°C day and 16°C night. Light and temperature regimes were maintained for the entire study, unless otherwise stated. Plants were watered daily to field capacity and fertilized every 14 days using 20:20:20 (N-P-K) (Complete Plant Products, Brampton, Ont.). At the 4 leaf stage, plants were transferred to 4°C for 6 weeks to complete vernalization and then transferred to a programmable growth cabinet (Conviron PGW 36, Winnipeg, Manitoba) under 10°C (day) and 6°C (night), to synchronize bud development. Buds that had started to mature and change color (from dark green to yellow), indicating impending anthesis, were chosen for crossing.

A total of 9 reciprocal crosses were completed (18 plants). Individual buds were opened using ethanol-sterilized forceps. Sepals, petals and anthers were removed from the female flower prior to dehiscence of pollen. Stigmas were pollinated with mature pollen from the appropriate donor parent and enclosed in pollination bags until silique elongation was apparent (evidence of embryo fertilization). This ensured that contamination from any air-borne pollen would not occur. Once all reciprocal crosses had been completed, plants were returned to the greenhouse to allow F_1 seed to mature and be harvested.

2.2.2. Microspore Culture

F_1 seed was planted and grown as described above. Plants used for microspore culture were maintained in the growth cabinet after vernalization at 10°C day and 6°C night to synchronize bud development. Buds to be used for microspore embryo production were 3-5 mm in length and were collected from healthy plants just prior to anthesis. Buds were placed into a wire strainer and surface sterilized in 100 mL 7% sodium hypochlorite for 15 minutes, then rinsed three times in 150 mL of sterile water for 5 minutes each. All subsequent work was completed in a laminar flow bench using autoclaved solutions and instruments to ensure sterile conditions were maintained. Buds were transferred to a mortar and gently crushed in 1-3 mL of B5 medium containing 13% sucrose (Coventry et al., 1988) to release the microspore cells. The suspension was filtered through a 0.44 µm nylon filter into a 15 mL Falcon tube (Fisher), and the nylon filter was rinsed twice with 1 mL aliquots of B5 medium. The volume in all tubes was then adjusted to 10 mL B5 medium prior to centrifugation in a table top centrifuge (Model IEC-HN-SII, Needam, Massachusetts) at 1000 rpm for 3 minutes. The supernatant was removed and the pellet was resuspended in 5 mL B5 medium containing 13% sucrose. Three more washes were completed with centrifugation at 800 rpm, 500 rpm and 500 rpm, respectively. The final pellet was suspended in NLN medium (13% sucrose) (Lichter, 1982). The volume of the suspension was adjusted to 1 mL for every original bud used (approximately 20 per isolation). The suspension was then poured into sterile petri plates (60 x 15 mm, Fisher) and sealed with Parafilm. Plates were heat shocked for 3 days at 37°C in the dark with no agitation. Plates were then transferred to a shaker (55-60 rpm) at room temperature in the dark for 4-6 weeks. Embryos were selected and plated onto solid B5 medium (3% agar) (Difco-Bacto), supplemented with 3% sucrose (Coventry et al., 1988) and placed on a light bench with a light intensity of 150 µmole $m^{-2}s^{-1}$, at room temperature to grow until roots and true leaves were apparent. Haploid plantlets were transferred to sterile soil and placed in a mist chamber under high humidity for 14 days to reduce desiccation and excessive stress, then were transferred to the greenhouse and allowed to grow until 3-5 mature leaves were apparent, at which time they were transferred to 4°C for 6 weeks for vernalization.

2.2.3. Fertility Assessment and Colchicine Treatment

Vernalized haploid plants were moved to the greenhouse and assessed for fertility. Bolting plants were analyzed for the presence of pollen as an indicator of spontaneous doubling. Where chromosome doubling had not occurred, up to 5 cuttings were taken from a single plant and dipped in powdered rooting compound (0.2% indole-3- butryic acid) (Plant Products Co., Bramlea, Ontario), and rooted in moist soil in a misting chamber. Rooted cuttings were removed and soil was washed from the roots. Roots were trimmed to 6 cm and foliage trimmed from shoots to approximately 20 cm. Roots were placed in an aqueous (3.14 g L^{-1}) colchicine solution according to Coventry et al. (1988), and placed under high light intensity (300 μmole $m^{-2}s^{-1}$) for 2 hours to maximize transpiration and colchicine uptake. Plants were thoroughly rinsed in water, potted into 15 cm pots and placed in the greenhouse. A single doubled shoot from each DH_1 line was maintained and bagged to obtain self-pollinated seed. All other shoots and plants were discarded.

2.2.4. Assessment of Freezing Tolerance

Freezing tolerance was assessed on plant material using an electrolyte leakage assay. Inherent freezing tolerance was assessed on nonacclimated material that was maintained in the greenhouse. Twenty-four randomly selected DH lines were assayed as a representative sample of the population of DH lines. Acclimation-specific freezing tolerance was determined on 2 parent lines, 6 F_1s, and 78 DH lines that had been exposed at 4°C for 6 weeks, as described for vernalization. Assessments were completed on all 78 lines (Appendix 6.1). The second and third leaves of 4 leaf stage plants and the apical leaves of bolting plants were harvested and washed in distilled water. One centimeter discs of leaf tissue were removed using a cork borer, being careful to avoid any veins. Discs were then placed on moistened filter paper (1 disc per filter) (Whatman) in small petri plates (2 filters per plate). Plates were placed in a programmable freezer and the temperature lowered to 0°C over a 1 hour period. A set of plates were removed at this temperature to act as the non-frozen control. The temperature was then lowered to -2.5°C over a 1 hour period and nucleation was initiated by touching the filter paper with a metal probe cooled in liquid nitrogen. Samples were maintained overnight at -2.5°C. The temperature was then lowered at a constant rate of -2.5°C/hr and samples were removed at 2.5°C intervals from -2.5°C to -18.5°C. As samples were removed, they were allowed to thaw at 4°C for at least 12 hours. Electrolyte leakage was determined by placing the thawed discs (including paper) in tubes containing 10 mL of double distilled water and shaking (45 rpm) overnight at room temperature. Freeze-induced leakage was determined by measuring conductivity using a radiometer (Model CAM 83, Bach-Simpson LTD, London, Ontario). Total ion leakage was determined after boiling samples for 3 minutes, cooling to room temperature, and shaking for at least 1 hour at 45 rpm. Ion leakage was measured in each case and injury was expressed as a percentage of total (boiled). The temperature at which 50% leakage occurred, termed TL_{50}, was used as a measure of plant viability at that particular temperature given that 50% leakage is equivalent to 99% cell death and consequent plant mortality (Sukumaran and Weiser, 1972; Boothe et al., 1995). Acclimation capacity was determined as the difference in freezing tolerance between nonacclimated and acclimated material on the 24 lines assayed for inherent levels of freezing tolerance (Appendix 6.1). Freezing tolerance was determined to a maximum level of –18.5°C, because of the mechanical constraints of the freezer.

2.2.5. Sample Size and Statistical Analysis

SAS version 6.03 was used to assess the significance of random effects on the distribution of freezing tolerance in the DH lines at the level of inherent freezing tolerance and acclimation-specific freezing tolerance. From the DH population, 24 lines were randomly selected for determination of inherent freezing tolerance. Analysis of inherent tolerance was completed using 2 replicates. Each replicate contained 5 plants/line (n=10). For each plant, leakage measurements were averaged from 3 discs at each of 5 different temperatures (0, -2.5, -5, -7.5 and –10°C). Analysis of acclimation-specific freezing tolerance was completed using 3 replicates. Each replicate contained 5 plants/line (n=15). For each plant, leakage measurements were averaged from 5 discs, taken at each of 5 different temperatures (0, -7.5, -12.5, -15 and –17.5°C). The resulting means were used to generate a graph, from which the TL_{50}s were determined. The difference between inherent (TL_{50}) and and acclimation specific freezing tolerance (TL_{50}) was the acclimation capacity. The design used was ANOVA univariate analysis using the general linear models (GLM) procedure on replicate by line to examine the random effect within each replicate. The line by line analysis was completed in the same manner and used to compare random effects between individual DH_1 lines as seen by differences in freezing tolerance. Coefficient of variance was calculated using (root mean squared error/mean) as a second measure of significance in the test.

Association of phenotype with individual DNA clones was completed using Fishers exact (two-tailed) test for association. Calculated numbers indicate the percentage of association that is attributed to chance alone.

2.2.6. DNA Extraction

At the 4 leaf stage, young leaf tissue was excised, flash frozen in liquid nitrogen and stored at -80°C until used for genotypic assessment. DNA was extracted from approximately 5g of tissue according to Dellaporta et al. (1983). Tissue was ground to a fine powder in a mortar and pestle under liquid nitrogen and then ground in 10 mL of extraction buffer (100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 50 mM EDTA, pH, 8.0, to which 7 µL of 144 mM of 2-mercaptoethanol was added fresh). To the slurry, 1 mL of 20% SDS was added and the sample was mixed thoroughly, then 70 µL of 50 mg/mL proteinase K was added to each sample, mixed, and the sample was incubated at 65°C for 1 hour. To this, 2 mL of 5 M potassium acetate was added and sample mixed gently, but thoroughly, then placed on ice for 15 minutes. Samples were then centrifuged for 10 minutes at 10,000xg and the supernatant filtered through a layer of Miracloth (Cal-Biochem). Samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by centrifugation for 10 min at 10,000xg. The aqueous phase was removed and placed in a clean tube and precipitated with 0.6 volumes of isopropanol. DNA was then spooled out of solution, rinsed three times in 70 % ethanol, air dried and dissolved in TE8 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). Samples were treated with RNAse according to Sambrook et al. (1989). Five molar NaCl was added to a final concentration of 2.5M (Fang et al., 1992). DNA was precipitated with 2 volumes of 95% ethanol, placed at -20°C for 30 minutes and then centrifuged at 4,500xg for 10 minutes at 4°C. The pellet was rinsed 3 times in 70% ethanol and dried. Extracted DNA was resuspended in a minimal volume of TE8 and quantified using ethidium bromide fluorometry (Karsten and Wallenberger, 1977). Briefly: 2 µL of DNA was mixed with 2.4 mL phosphate buffered saline (PBS) (170mM NaCl, 3.3mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4, pH 7.2) and 0.1 mL ethidium bromide (100 µg/mL). Samples were read on a Varian SF-330 spectrofluorometer with excitation at 360 nm and absorbance at 580 nm and were compared with a calibration curve constructed using known quantities of Lambda DNA.

2.2.7. Probe Preparation

Probes used in this study came from a number of sources. The genomic clones used were isolated from a genomic library of *Brassica napus* (Table 2.2). They were a gift from Dr T. C. Osborn. BN115 and BN28 are clones from a low temperature cDNA library of *Brassica napus* cv. Jet Neuf. The BN clones were a gift from Dr. J. Singh. The cold-regulated clone (cor6.6) came from a cDNA library of cold acclimated *Arabidopsis thaliana* and was a gift from Dr. M. Thomashow. All the clones had T7 and T3 priming sites adjacent to the multiple cloning site of the clone. This allowed for amplification of the inserts using PCR. O.1 µg of template was used in all amplifications using the enzyme Taq DNA poymerse (Promega). T7 and T3 primers were purchased from Promega. PCR was completed using a Techne PHC-2 thermocycler, using the fastest possible ramping times. DNA was initially denatured at 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 39°C for 1 minute and 72°C for 2 minutes. A final elongation at 72°C for 10 minutes ensured all products would be of equal length. Purity of amplification was assessed by electrophoresis of 2 µL of sample in 0.8 % TAE agarose gel and visualized by ethidium bromide staining according to Sambrook et al. (1989). Samples were purified using G-50 Sephadex in a spun column according to Sambrook et al. (1989). Samples were quantified by fluorometry as above and 75-100 ng was used in for each labeling reaction.

2.2.8. Southern Blotting

Genomic DNA (20 µg) was restricted according to the manufacturer's instructions using Eco R1 or Hind III (Boeringer Mannheim). Digested samples were separated on an 0.8% agarose gel in 1X TAE at 80 volts for 3 hours (Sambrook et al., 1989). Equal loading of restricted samples was assessed visually using ethidium bromide fluorescence of separated samples. Gels were capillary blotted onto Zeta-probe nylon membranes (Bio-Rad Laboratories, 3300 Regatta Blvd., Richmond, CA) using the semi-dry method of Rutledge et al. (1985). DNA was cross-linked to the membrane by illumination at 254nm then baked at 80°C for 30 minutes under vacuum (Southern 1975). Blots were prehybridized and hybridized according to the membrane manufacturer's instructions using 50% formamide at 43°C. Membranes were hybridized using a $^{32}P\alpha dCTP$ (Amersham) labeled probe (Random Priming Kit BRL #55567656) and rinsed at room temperature as follows: once for 2 min in 2X SSC and 0.1% SDS; once for 10 min in 1X SSC and 0.1% SDS; once for 10 min in 0.5X SSC and 0.1% SDS; and a final wash at 65°C for 10 min in 0.1X SSC and 0.1% SDS. Membranes were exposed to XAR-5 film (Kodak) with intensifying screens at -80°C for 2-5 days. Individual lines were scored as being freezing tolerant (+) or scored as being freezing sensitive (-). Freezing tolerant (+) lines had TL_{50}s greater than or equal to -14.5°C. Freezing sensitive (-) lines had TL_{50}s less than or equal to -9°C. All plants falling between tolerant and sensitive were scored as intermediates (Appendix 6.1).

2.3. RESULTS

2.3.1 Microspore Embryogenesis

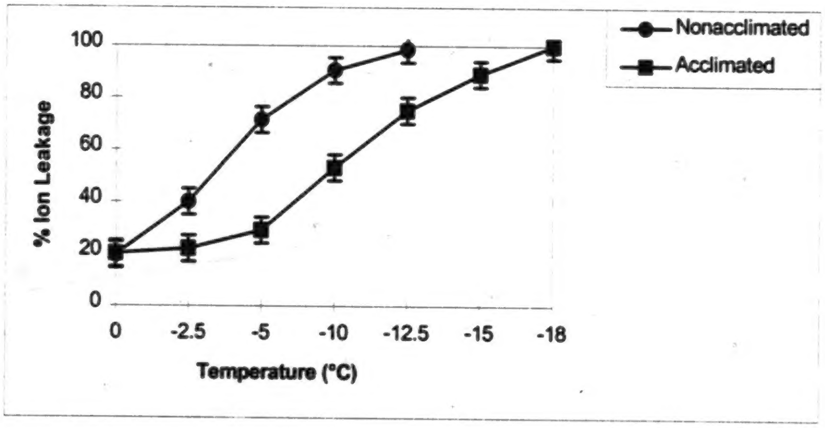
Approximately 100 DH lines were derived from tissue culture, of which 78 lines were maintained for further analysis. Spontaneous doubling accounted for 3% of the doubling that had occurred (Appendix 6.1). The remainder of the lines were doubled with colchicine. Aside from fertility, no other phenotypic differences were apparent between any of the colchicine treated material and the spontaneous diploids used in this study. There was no maternal effects exhibited by any of the lines tested.

2.3.2. Freezing Tolerance and Acclimation Capacity

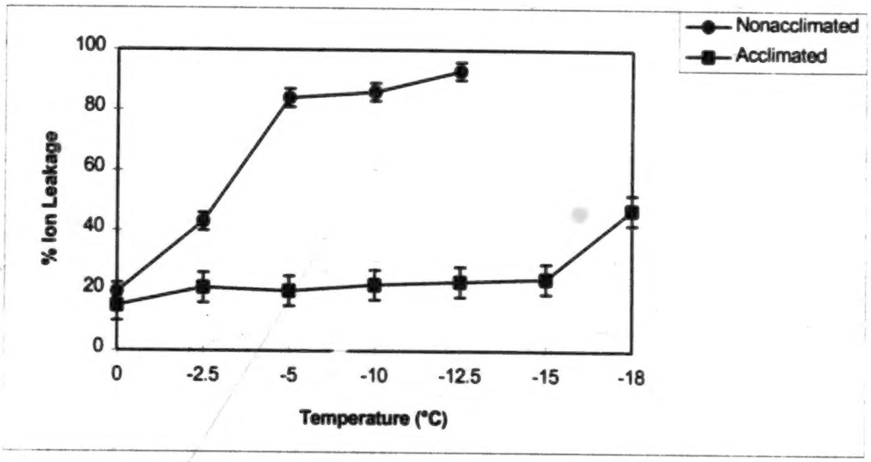
Parents, F_1 and DH lines were assessed for inherent and acclimation-specific freezing tolerance, based on freeze-induced ion leakage (Fig 2.1 and Appendix 6.1). The difference between inherent and acclimation-specific freezing tolerance represents the acclimation capacity of the line. The acclimation capacity of 24 randomly selected lines ranged from a low of 1.0°C to a high of 14°C (Appendix 6.1). Results indicated there are 3 distinct aspects of freezing tolerance present in these DH lines: inherent freezing tolerance; acclimation-specific tolerance; and acclimation capacity.

Figure 2.1. Inherent and Acclimation-Specific Freezing Tolerance in Representative DH$_1$ Lines of *B. napus*. Assessment of ion leakage in nonacclimated and acclimated leaf tissue. A) line 8-117, B) line 3-116 and C) line 6-200. The values represent the mean +/- S.E., n=10 and n=15 for inherent and acclimation-specific tolerance, respectively.

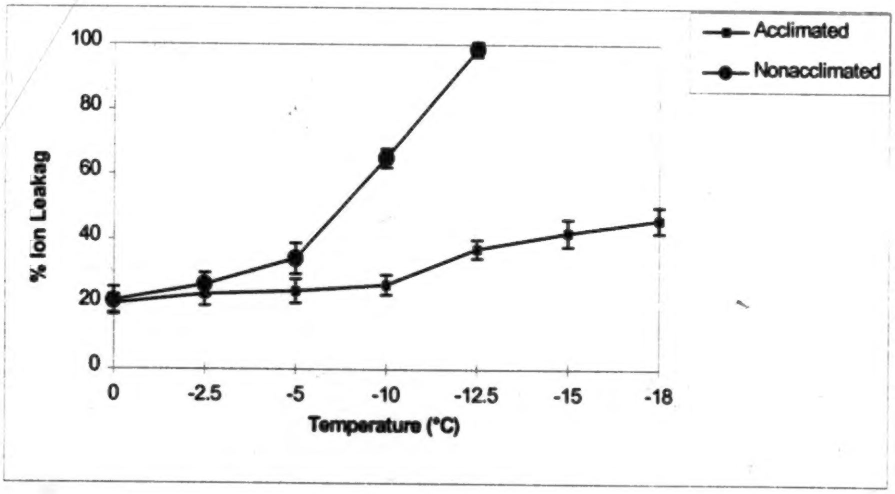
A) Line 8-117



B) Line 3-116



C) Line 6-200



2.3.3. Distribution of Acclimation-Specific Freezing Tolerance

Freezing tolerance was assessed on acclimated material to gain insight into the phenotype of individual DH lines as compared to the original parents. TL_{50}s were calculated for each parent. Cascade was found to be freezing tolerant (-15.5°C) while Rebel was freezing sensitive (-7.5°C). Distribution of acclimation-specific freezing tolerance in the DH lines showed a continuous trend that ranged from -3.0°C to greater than -18°C (Fig 2.2). There appeared to be some peaks in the distribution, but nothing to indicate specific gene action. There appeared to be a large proportion of transgressive segregation, which favored a level of freezing tolerance greater than that of the tolerant parent, Cascade. As expected, the distribution of the curve did not conform to any Mendelian ratio and represents the effect of many genes acting on the phenotype of freezing tolerance.

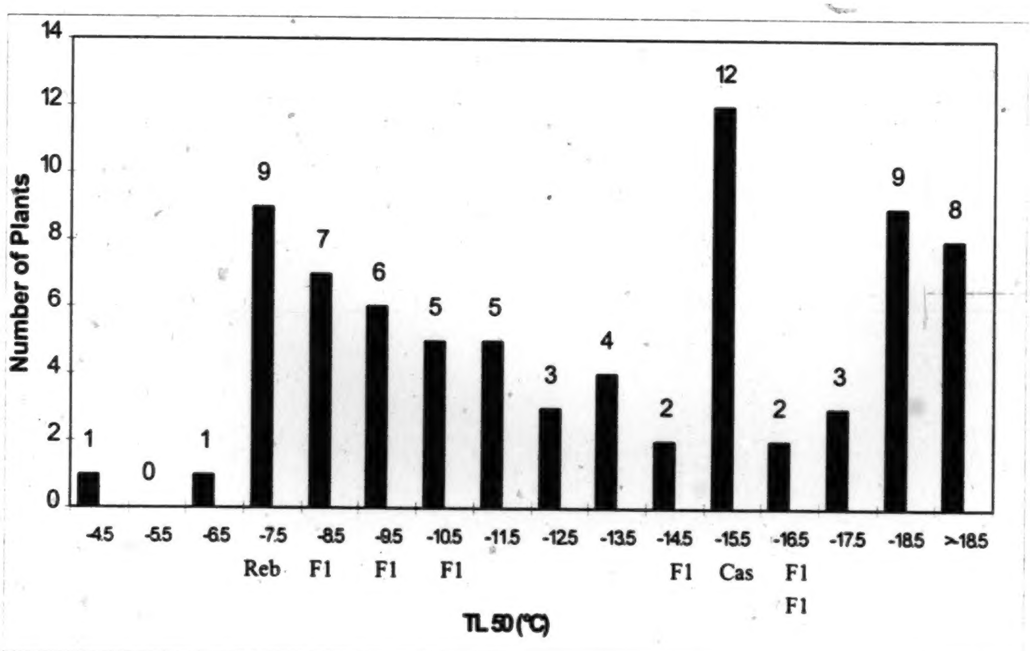
2.3.4. Statistical Analysis

Comparison of random effects in REPLICATE X LINE using ANOVA univariate analysis revealed no significant difference between replicates (Table 2.1). However, when comparing random effects of LINE X LINE, highly significant differences were apparent. A correlation of variance value of 5.8 % (LINE X LINE) confirmed that significant levels of variation exist.

Table 2.1. Statistical Analysis of Acclimation-Specific Freezing Tolerance. ANOVA univariate analysis using the general linear model (GLM), using SAS ver 6.03 (SAS Institute Inc 1988). Line and Replicate were analyzed as random. Analysis was compared on Replicate x Replicate and Replicate x Line arrangement.

	Degree of Freedom	Sum of Squares	F Value	Mean Square	Pr > F	Coefficient of Variance
REP	**2**	**0.13**	**0.1**	**0.06**	0.89	**n/a**
LINE	**77**	**3,128**	**69.2**	**41.8**	<0.01	5.8% (RepxLine)
ERROR	**154**	**93.2**	**n/a**	**0.6**	**n/a**	**n/a**

Figure 2.2. Distribution of Acclimation-Specific Freezing Tolerance. Segregation of freezing tolerance based on TL$_{50}$s of 72 individual lines of *B. napus* (Includes 2 parent lines, 6 F$_1$, and 70 DH lines).



In comparing reciprocal crosses, differences in the level of freezing tolerance could not be attributed to maternal effects. Freezing tolerance appeared to segregate randomly throughout the DH_1 lines regardless of the direction of the cross.

2.3.5. Correlation Between Inherent and Acclimation-Specific Freezing Tolerance

Correlation analysis was completed between nonacclimated and acclimated tissue to examine the possibility that inherent freezing tolerance could be used as a tool in predicting a line's acclimation capacity (Fig 2.3). Acclimation capacity is the ability of a DH line to gain freezing tolerance over its inherent freezing tolerance. The results showed that neither acclimation-specific freezing tolerance nor acclimation capacity can be predicted using inherent freezing tolerance as an indicator.

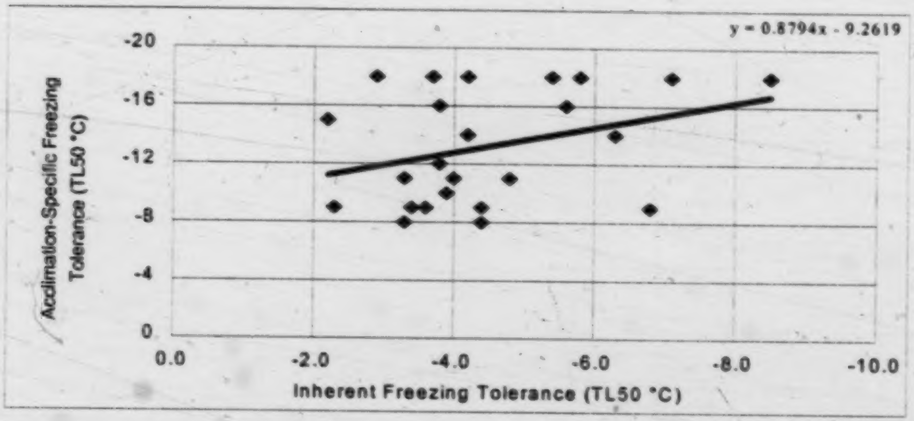
2.3.6. Genotypic Analysis

Genotypic analysis was completed amongst the DH lines segregating for freezing tolerance. Use of low temperature-induced clones (BN28, BN115 and cor6.6) revealed no polymorphism between the parents, or any of DH progeny

(Fig 2.4). Genomic screening showed that 2 of 8 genomic clones, identified by Teutonico et al. (1995) as being linked to freezing tolerance, had polymorphic differences between parents. The polymorphism was complemented in the F_1 and segregated amongst the DH lines analyzed (Fig 2.5 A and B) (Table 2.2). WG1G6 did not appear to be associated with freezing tolerance, whereas EC2E5 appeared to be associated with freezing tolerance.

Figure 2.3. Correlation Between Inherent Freezing Tolerance and Both Acclimation-Specific Freezing Tolerance and Acclimation Capacity in DH Lines. **A)** Correlation between inherent and acclimation-specific freezing tolerance. **B)** Correlation between inherent freezing tolerance and acclimation capacity. Twenty four DH lines of *B. napus.* were assessed. (acclimation capacity = acclimated FT- inherent FT).

A)



B)

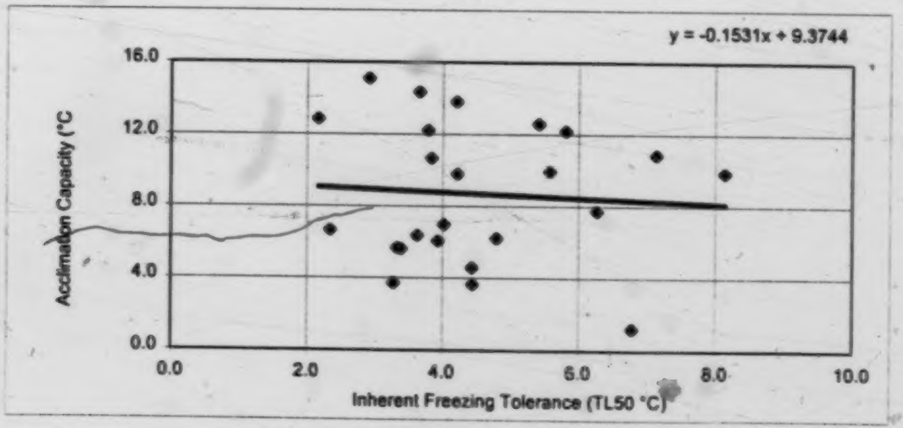


Figure 2.4. RFLP Analysis of Parents and DH Lines. Representative blot probed with cDNA clone BN28 from *Brassica napus*. Cascade (C), Rebel (R), F_1 and DH_1 lines. Acclimation-specific freezing tolerance was denoted as: (+) freezing tolerant and (-) freezing sensitive.

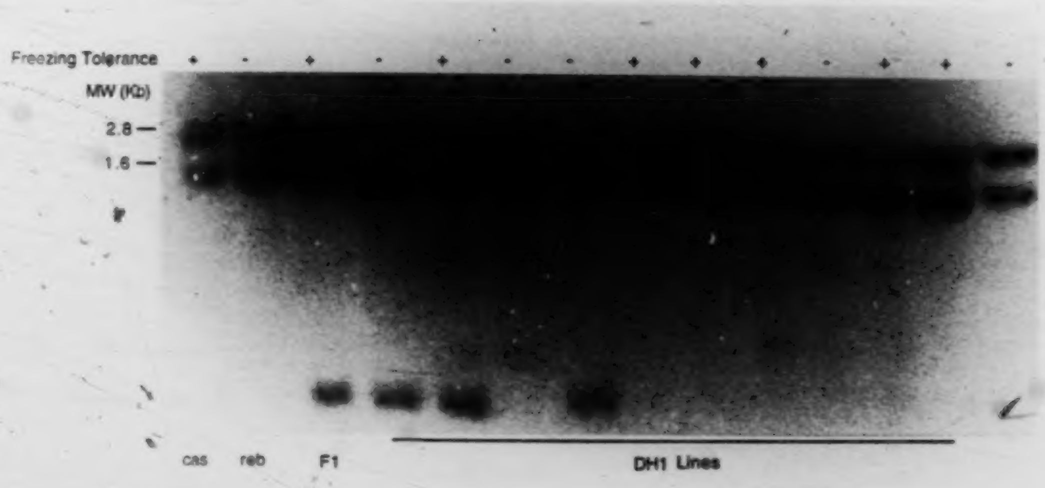
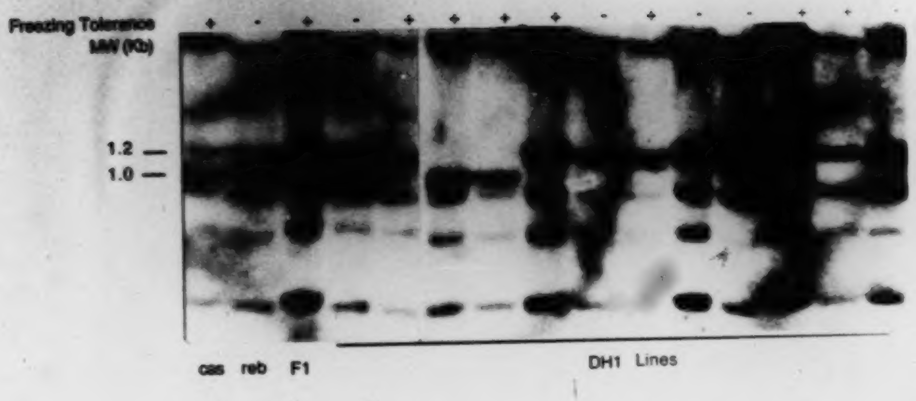


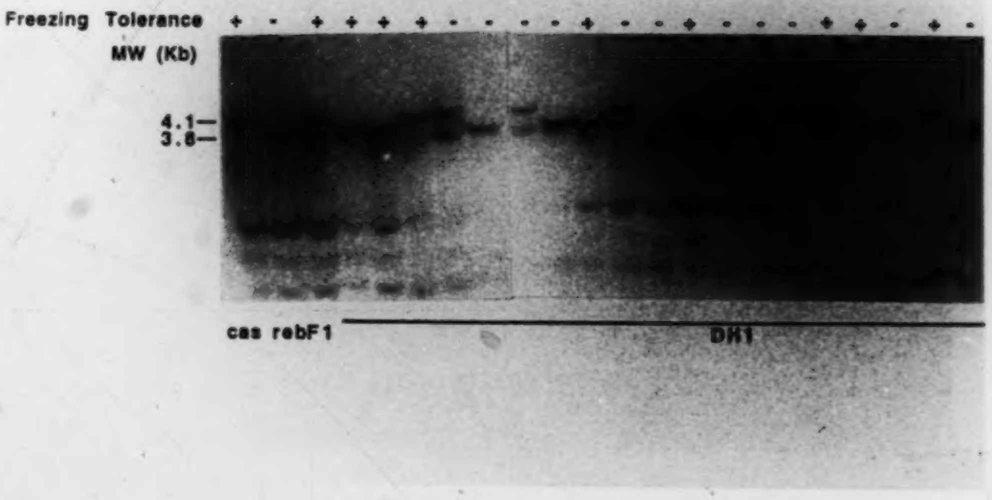
Table 2.2. Analysis of Association in DH lines. Probes from low temperature-induced cDNA clones (BN28 and BN115 from *B. napus* and cor6.6 from *Arabidopsis thaliana*) and genomic clones from either a non-specific cDNA library (EC clones) or genomic library of *B. napus* (WG and TG clones) (From T. C. Osborn) were used in this study. Association calculated using Fishers exact test (2-tailed) (0 = good association and 1 = no association).

Clone	Source	Polymorphic	Fragment size Kb	Association (P) value	Sample Size (n)
BN28	B. napus	No	n/a	n/a	22
BN115	B. napus	No	n/a	n/a	22
Cor6.6	A. thaliana	No	n/a	n/a	22
EC2E5	B. napus	Yes	1	0.007	32
WG1G6	B. napus	Yes	2.6	1.0	32
WG1G3	B. napus	No	n/a	n/a	32
WG1F6	B. napus	No	n/a	n/a	32
WG4H3	B. napus	No	n/a	n/a	32
EC4H3	B. napus	No	n/a	n/a	32
TG5B2	B. napus	No	n/a	n/a	32
TG1C8	B. napus	No	n/a	n/a	32

Figure 2.5. RFLP Analysis of Parents and DH Lines. A representative blot probed with cDNA clone EC2E5 and genomic clone WG1G6 A) Blot restricted with Eco R1 enzyme and probed with EC2E5.and B) restricted with HIND III and probed with genomic clone WG1G6. (c) Cascade, (r) Rebel, F_1 and DH_1 lines. Acclimation-specific freezing tolerance was denoted as:(+) freezing tolerant and (-) freezing sensitive.


Freezing Tolerance
+ - + - + + + + - + - - + + -
MW (Kb)
1.2
1.0
cas
reb
F1
DH1 Lines

B) restricted with Hind III and probed with genomic clone WG1G6. (cas) Cascade, (reb) Rebel F_1 and DH lines. Acclimation-specific freezing tolerance was denoted as: (+) freezing tolerant and (-) freezing sensitive.



2.4. DISCUSSION AND CONCLUSIONS

Through microspore embryo technology, a population of homozygous DH lines in *Brassica napus* has been developed, which can now be used to dissect the complex physiological and molecular controls required for inherent and acclimation-specific freezing tolerance. In addition, this freezing tolerant material may be useful for the development of hardier canola cultivars.

Ion leakage was used in the present study as an accurate assessment of inherent and acclimation-specific freezing tolerance. One of the current methods to assess winter hardiness in the *Brassicaceae* is to use replicated winter survival tests on populations of plants (LT_{50}) (Zenon Lisieczko, Limagrain Canada Seeds Inc. personal communication). This approach measures freezing tolerance in combination with all other aspects of winter hardiness. It is subject to variation from the impact of environmental conditions. This creates a need for a consistent laboratory test that accurately reflects freezing tolerance levels in nature. To assess ion leakage as an accurate measure of freezing tolerance in the Brassicas, Andrews and Morrison (1992) compared freezing tolerance survival of whole plants using (TL_{50}) to ion leakage data (LT_{50}) in the *Brassicas*. They concluded that ion leakage (LT_{50}), although crude, could be used as an accurate indicator of a plant's ability to survive freezing temperature. Previous work in our lab has shown that ion leakage correlates well with whole plant survival if it is assumed that TL_{50} is equal to 99% cell death and consequent plant mortality (Singh, 1995). Freezing tolerance was assessed in all of the DH lines using the results of Andrews and Morrison (1992) and Singh (1995) as a guide. The results of this work may still require testing of DH lines in the field, to compare ion leakage with whole plant survival in this system. Only then will the validity of this laboratory test for the *Brassicaceae* be known. Also, those transgressive DH lines which failed to reach their TL_{50} because of the mechanical constraints of this system, may require additional testing before assigning an accurate freezing tolerance value.

The developmental age of plant material selected for freeze testing is important to attain accurate ion leakage results. Cox and Levitt (1976) and Boothe et al. (1995) both showed that older leaves on a plant tend to have reduced levels of freezing tolerance relative to younger leaves. For this study, we used the second and third leaves from a four leaf stage plant. These leaves are initiated at the same time and are of similar physiological and developmental age. These leaves also provided enough plant material for an accurate assessment of freezing tolerance from individual plants as well as plant lines.

Freezing tolerance in plants is composed of two major components: freezing tolerance in the nonacclimated state (inherent tolerance); and the ability to gain freezing tolerance through the process of acclimation (acclimation-specific tolerance) (Stone et al., 1993). The relative difference between inherent and acclimation-specific levels of freezing tolerance is the acclimation capacity of the line. The genetic nature of inherent and acclimation-specific freezing tolerance and acclimation capacity has not been resolved. Results from this work (Fig 2.2a) indicate that inherent freezing tolerance and acclimation-specific freezing tolerance are not correlated. Similarly, inherent freezing tolerance is not correlated with acclimation capacity (Fig 2.2b). Concurrent with our study, Teutonico et al. (1995) and Stone et al. (1993) showed that nonacclimated freezing tolerance and acclimation-specific freezing tolerance were not correlated in *B. napus* and in *Solanum commersonii* (potato) respectively. The work completed by Teutonico et al. (1993) dealt with a winter by spring cross. As this study deals with a winter by winter, the results may differ from those described by Teutonico et al. (1995).

The DH lines being used in this study are stable and no longer segregating for the freezing tolerance. To be able to complete the required replicates needed for freezing tolerance assessment, several plants had to be grown, spanning 4 generations of self-pollination. Similar data were collected on parent lines as they are not DH lines, but derived from conventional breeding (open polinated) and selection practices. Although genomic variability exists in the parental cultivars, variability of freezing tolerance has not been observed in these cultivars (Boothe et al., 1995) Statistical assessment of inherent freezing tolerance and acclimation-specific freezing tolerance of DH lines is another indication of the stability of the DH lines (Table 2.1). The stability of the population is important if characterization of these lines for their unique freezing tolerance traits is to be successful.

It is important to check for potential differences between and within lines. Coefficient of variance (cv) expresses the standard deviation as a percentage of the mean (Devore and Peck 1990). In this system coefficient of variance was used to examine the relative variability within and between DH lines. According to Gomez and Gomez (1984) a cv value equal to or less than 12% in an agronomic system indicates a low degree of variation and further strengthens the significance of the data represented in the test. Thus, the results of the present study (coefficient of variance = 5.8%) indicates that each line represents a unique phenotype in terms of acclimation-specific freezing tolerance. Segregation of acclimation-specific freezing tolerance was one objective addressed in this study. It was possible to maximize the potential segregation of this trait in the progeny, by using 2 parent lines with an 8°C difference in freezing tolerance.

Segregation analysis based on TL_{50} (Table 2.2) agrees with literature published concurrently with this study (Stone et al., 1993; Teutonico et al., 1993; Galiba et al., 1995), indicating that freezing tolerance is a complex trait. The data set, reveals a continuous distribution of freezing tolerance as there is nothing to suggest that single gene Mendelian principles are applicable.

Interestingly, a large percentage of transgressive segregation was apparent in the present study, with 16 of 70 DH_1 lines (23%) displaying levels of tolerance that exceeded that of the tolerant parent Cascade (-15.5°C). Similar results were reported by Teutonico et al. (1995) who found 77% of segregants showing freezing tolerance in excess of that of the tolerant parent. This suggests that optimum gene combinations have occurred, with positive alleles coming from each parent.

Genetic analysis in this system has confirmed the complex nature of freezing tolerance. It has also revealed that the genetics can be optimized at the level of both inherent and acclimation-specific freezing tolerance on an individual plant basis. This makes the population ideal for genetic mapping studies. Previous work uncovered 4 regions, or linkage groups associated with freezing tolerance in *Brassica rapa* (Teutonico et al., 1995; see Appendix 6.2). I have used the markers to examine possible freezing tolerance linkage groups within my system (Fig 2.4 and Table 2.2). There are subtle differences between the results of the present study and those of Teutonico et al. (1995). They (Teutonico et al., 1995) showed that both EC2E5 and WG1G6 are linked to freezing tolerance in B. rapa (Appendix 6.2). Their work failed to link any regions in *B. napus*. In my system, several clones tested, including WG1G6, do not appear to be assocaiated with freezing tolerance, whereas clone EC2E5 appears to be associated with freezing tolerance (Table 2.2). The differences in RFLP patterns may reflect the differences in the parentage of the initial crosses made in the two studies. Until all regions have been mapped thoroughly, all we can conclude is that our system may have the capacity to define important linkage groups in terms of freezing tolerance in *Brassica napus*.

In this study, a number of cDNA clones that are induced by exposure to low temperature have been examined (Table 2.2). Linkage to the phenotype would provide evidence for an association with freezing tolerance. Thus, it is interesting to note that only one cDNA, cor 6.6, has been shown to be associated with freezing tolerance, and this was in *B. rapa* (Teutonico et al., 1995). It was not linked, or associated with either the *B. napus* population of Teutonico et al. (1995) or the population used in the present study.

The co-segregation of a phenotype with a genotype is one criterion that can be used to suggest a gene function. Another approach is to examine the physiological or biochemical role of the gene product. It is hoped that the plant lines developed in the present study may be useful for testing gene function.

Increased freezing tolerance would be of considerable value in new winter cultivars. The present study produced plant lines with acclimation-specific freezing tolerance in excess of –18°C. This represents an improvement of at least 3°C. As these lines are derived from canola quality material, there is likely the potential to increase freezing tolerance in winter canola.

There may also be the potential to increase freezing tolerance in spring canola. Spring type canola cultivars often suffer from late spring frosts while plants are at the seedling stage and then in the fall during maturation. If inherent tolerance of –8.5°C (measured in the present study) could be transferred to spring canola, significant progress could be made in developing tolerance to these frosts.

In summary, a population of DH lines has been produced, capable of resolving the phenotypic and genotypic differences associated with inherent and acclimation-specific freezing tolerance in *Brassica napus*. Answers pertaining to the genetic components of freezing tolerance may be found by analyzing those DH lines which fall into 1 of 4 categories: i) poor inherent tolerance and little ability to acclimate; ii) Poor inherent tolerance but the ability to acclimate; iii) high levels of inherent tolerance but little acclimation ability; and iv) high levels of both inherent and acclimation-specific tolerance. The process outlined herein provides a window through which the genetics of freezing tolerance may be further characterized and defined in the *Brassicaceae*.

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CHAPTER 3

Genetic Relationship and Expression Patterns of BN28, A Low Temperature Induced Gene in *Brassica napus*

3.1. INTRODUCTION

Molecular maps have been produced in a number of *Brassica* species including *B. napus* (Landry et al., 1991; Ferreira et al., 1994), *B. rapa* (Song et al., 1991; Chyi et al., 1992) and *B. oleracea* (Slocum et al., 1990; Kianian and Quiros, 1992; Landry et al., 1992). The use of quantitative phenotypic markers has been a mainstay in terms of selection criteria for development of new crop species (King, 1990). Recently, molecular markers have been used to map quantitative trait loci (QTL) in *B.napus* (Teutonico et al., 1995). These linkage maps allow for early screening and manipulation of quantitative traits based on marker assisted selection (Tanksley et al., 1988). Species-specific maps can also be used to search for homologues or duplicated. From these, models of gene function can be created and their phenotypic roles determined.

The original surveys on evolution and inheritance patterns in *Brassicaceae* were completed by U (1935). U showed that the *Brassicaceae* are comprised of three parental diploid genomes: *B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC). Allotetraploids arise through interspecific association between any two of the diploid parents as outlined by U (1935). The ancestry of the allotetraploid *B. napus* (AACC), can be traced back to an interspecific cross between the two diploid parents *B. rapa* (AA) and *B. oleracea* (CC) (U, 1935; Erickson et al., 1983; Song and Osborn, 1992). Similar relationships exist for *B. carinata* (BBCC) and *B. juncea* (AABB), and the distantly related diploid species *Sinapsis arvensis* (SS) and *Sinapsis alba* (dd) (U, 1935).

Molecular markers have been used in the search for cold-acclimation-responsive loci (Cai et al., 1994). Identification of low temperature markers in the *Brassicaceae* would assist in the breeding and selection of lines capable of surviving freezing conditions. This could eventually lead to extension of canola growing areas into regions that are too cold or the growing season too short. The difficulty with this approach is that genes for quantitative traits can act separately or epistatically. Furthermore, their inheritance patterns can be complex (Song et al., 1995).

Cold acclimation is the result of complex morphological, physiological, molecular and biochemical changes (Singh and Laroche, 1988; Alberdi and Corcuera, 1991). Weiser (1970) was one of the first to demonstrate that exposure to low temperature and the resultant cold acclimation involve changes in gene expression. Subsequently, the quest for genes regulated by low temperature has led to the discovery of numerous genes from several plant species including *Spinacia oleracea* (Gut, 1990; Nevin et al., 1994*), Medicago sativa* (Laberge et al., 1993; Monroy et al., 1993*), Hordeum vulgare* (Cativelli and Bartels, 1990), *Arabidopsis thaliana* (Kurkela and Franck, 1990; Gilmour et al., 1992; Lin and Thomashow, 1992; Palva, 1994), *Triticum avesetivum* (Guo et al., 1992), and *Brassica napus* (Orr et al., 1992; Weretilnyk et al., 1993; Genebank, 1994).

Kurkela and Frank (1990) first isolated a low temperature induced gene from *Arabidopsis thaliana*, termed kin1. Molecular analysis showed the gene was induced by low temperature as well as treatment with exogenous ABA or osmotic stress. Orr et al. (1992) isolated a similar low temperature induced gene from *B. napus* (cv. Jet Neuf) termed BN28. Nucleic acid comparison of BN28 to Kin1 revealed a 77% homology of nucleic acids in the coding regions of the two genes. Recently, a homologous gene to BN28 was isolated from *B. rapa* (brkin1)(Genbank, spring 1994).

I was interested in determining the occurrence and inheritance of BN28 within the *Brassicaceae* family. As *B. napus* is an allotetraploid, the gene encoding BN28 may be present in some form within all the Brassica species as outlined by U (1935). The objective of the present study was to gain insight into the number of BN28 homologues present, from which diploid parent are the gene/s inherited, and how these genes are expressed in the *Brassicaceae*. Elucidation of the inheritance of BN28 may provide information assisting in the identification of and selection for improved low temperature stress tolerance among the *Brassicaceae*, while expression studies may provide insight into the function of BN28 *in vivo*.

3.2. MATERIALS AND METHODS

3.2.1. Plant Material

Seed of individual species were planted in pots containing a soil mixture of sand, peat and vermiculite (1:1:1) and grown under greenhouse conditions with a 16 hour-photoperiod augmented with High Intensity Discharge (HID Sylvania Canada) sodium vapour lights to maintain a minimum light intensity of 275-300 mE s^{-1} m^{-2}. At the four leaf stage, control tissues were excised, flash frozen in liquid nitrogen and stored at -80°C. Remaining plants were transferred to 4°C and a similar light regime for cold acclimation. After 14 days of acclimation tissues were collected, flash frozen in liquid nitrogen and stored at -80°C to be used for RNA and protein analysis.

3.2.2. DNA Extraction

DNA was extracted from approximately 5g of young leaf tissue by the method of Dellaporta (1990), with the following modifications: prior to isopropanol precipitation, samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged for 10 minutes at 10,000xg. Prior to ethanol precipitation, 5M NaCl was added to a final concentration of 2.5M (Fang et al., 1992). Samples were placed at -20°C for 30 min, pelleted at 4,500xg for 10 min at 4°C, rinsed three times in 70% ethanol, and dried. Extracted DNA was resuspended in a minimal volume of TE8 (10mM Tris-HCl pH 8, 1mM EDTA) and quantified using ethidium bromide fluorometry (Karsten and Wallenberger, 1977): 2ml of DNA was mixed with 2.4mL phosphate buffered saline (PBS) (170mM NaCl, 3.3mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 pH 7.2) and 0.1mL ethidium bromide (100µg/mL). Samples were read on a Varian SF-330 spectrofluorometer with excitation at 360nm and absorbance at 580nm. Values were then compared to a calibration curve constructed using known quantities of Lambda DNA.

3.2.3. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed using 1μg of target DNA and Taq DNA polymerase (Promega)(Saiki et al., 1988). Amplification was carried out on a Techne PHC-2 thermocycler using the fastest possible ramping times. Primers specific to the 5' and 3' ends of the coding region of BN28 were used (BN28 forward primer 5'ATGTCAGAGACCAACAAGAAT3', and BN28 reverse primer 5'GTCTTGTCCTTCACGAAGTT3'). DNA was initially denatured at 95°C for 4 minutes, immediately followed by 35 cycles at 95°C for 1 minute, 53°C for 1 minute and 72°C for 2 minutes, with a final elongation for 10 minutes at 72°C. Amplified products were separated on 1.4% agarose gels in 1X TAE at 5V/cm and visualized by ethidium bromide staining according to Sambrook et al. (1989).

3.2.4. Southern Blotting

20 μg of genomic DNA was digested according to the manufacturer's instructions using; Eco R1 (BRL) and Pvu 2 (Boehringer Mannheim). Digested

samples were separated on 0.8% agarose in 1X TAE at 80 volts for three hours. Gels were capillary-blotted using the semi dry method of Rutledge et al. (1985), onto Zeta-probe nylon membranes (Bio-Rad Laboratories, 3300 Regatta Blvd., Richmond, Ca 94804). DNA was cross linked to the membrane by illumination at 254nm followed by incubation under vacuum at 80°C for 30 minutes. Blots were prehybridized and hybridized according to the membrane manufacturer's instructions using 50% formamide at 43°C. Membranes were hybridized with a ^{32}PαdCTP(Amersham) labelled BN28 cDNA probe (Random Priming Kit BRL #55567656) and rinsed at room temperature as follows: once for 2 minutes in 2X SSC and 0.1% SDS; once for 10 minutes in 1X SSC and 0.1% SDS; once for 10 minutes in 0.5X SSC and 0.1% SDS; and a final wash at 65°C for 10 minutes in 0.1X SSC and 0.1% SDS. Membranes were exposed to XAR-5 film with enhancing screens at -80°C for 2-5 days.

3.2.5. Total RNA Extraction

Total RNA was extracted from control and acclimated tissue (4°C for 14 days) using the hot phenol method of Verwoerd et al. (1989). RNA was quantified using ethidium bromide fluorometry as above (Karsten and Wallenberger, 1977). Fluorometric values attained for RNA were multiplied by a

DNA to RNA conversion factor of 2.17 (Karsten and Wallenberger, 1977). Thirty µg of total RNA was separated on 1.4% denaturing agarose gels according to Sambrook et al. (1989). Gels were rinsed briefly in 1X SSC and transferred to Zeta-Probe membranes (Bio-Rad Laboratories, 3300 Regatta Blvd., Richmond, Ca 94804) by capillary action using excess 1X SSC as the transfer buffer. RNA was cross-linked to the membrane by illumination at 254nm followed by incubation under vacuum at 80°C for 30 minutes. Membranes were Hybridized with a ^{32}PαdCTP(Amersham) labeled BN28 cDNA probe (Random Priming Kit BRL #55567656) washed at high stringency twice for 10 minutes in 0.1X SSC and 0.1% SDS at 60°C) and exposed to XAR-5 film for 6- 8 hours.

3.2.6. Protein Extraction and Immunoblotting

Total SDS soluble proteins were extracted from approximately 0.1g of tissue in 50mM Tris-HCl pH 8, 50mM NaCl, 1% SDS and 10mM PMSF according to Sambrook et al., (1989). Protein concentrations were determined using the bicinchroninic acid (BCA) method of Smith et al., (1985). Thirty µg aliquots were then mixed with an equal volume of 2X SDS loading buffer (Sambrook et al., 1989) and boiled for 5 minutes prior to loading. Samples were separated on a 15% SDS-polyacrylamide gel using a modified Tris-Tricine running buffer

(Shagger and von Jagow, 1987) at 30 milliamps (mA) until the tracking dye ran off the end. Proteins were transferred to 0.22 µm supported nitrocellulose membranes (Ba-S Schleicher and Schuell Keene, NH 0341) in carbonate buffer (Dunn, 1986) at 300 mA for 2 hours at 4°C. Blots were then reacted with BN28 specific antibody according to Boothe et al., (1995). The antibody was detected using alkaline phosphatase conjugated secondary antibody (Sigma) and visualized using the 5-bromo-4-chloro-3-indoyl phosphate-p-toluidine salt (BCIP) / p-nitro blue tetrazolium chloride salt (NBT) reagent system (Sigma).

3.3. RESULTS

3.3.1. PCR Analysis

Several seed sources were originally analyzed here to address the potential variability of species. (*B. rapa* had cv Eldorado and cv Tobin; *B. napus* had cv Legend, cv Cascade, cv Rebel and cv Westar, *B. oleracea* had cv Taipan and cv Golden acre; *B. Juncea* had cv Domo and cv Cutlas; *B. carinata, B nigra. S. alba and S. arvensis* had one line each). No differences were found within any species, hence only one cultivar for each species was represented in this study. The presence of possible BN28 homologues in the *Brassicaceae* was determined using the polymerase chain reaction (PCR) (Saiki et al., 1988), and primer sequences specific for the coding region of BN28 as reported by Orr et al. (1992) (Fig 3.1). Results indicate that a single fragment is amplified in all diploid species. The size of fragments were: *B. nigra*, 465 bp; *B. oleracea*, 425 bp; and *B. rapa*, 450 bp. Electrophoresis of a mixture of products from each of diploid species into resolvable fragments indicated each fragment is of unique size. *Brassica napus* is the only allotetraploid containing representative fragments from both original parents, *B. oleracea* and *B. rapa*. *Brassica carinata* contained the same size fragment as *B. rapa* and *B. juncea* contained the same fragment as *B. oleracea*. *S. alba* and *S. arvensis* amplified the same size fragment as *B. nigra*. The apparent band in the *B. napus* species and *S. alba* (approximately 570 bp, Fig 3.1) did not amplify consistently. It is assumed that they are artifacts, or possibly fragments with distant homology to the primers that were designed for BN28.

3.3.2. Southern Blot Analysis

Genomic differences within the *Brassicaceae* were analyzed in more detail using hybridization of a radiolabelled probe cDNA to Southern blots. Results indicate that each of the diploid parents contains a unique homologue of BN28 (Fig 3.2). Unique restriction profiles may reflect divergence that also appears to be associated with the different sized PCR fragments found in each species tested (Fig 3.1). Again, *B. napus* was the only allotetraploid to contain restriction fragments with the same molecular weights as both diploid parents. The other species tested, *B. carinata*, *B. juncea*, and *S. arvensis,* contained fragments similar to fragments from one diploid parent, in the same manner as the PCR analysis, except for Pvu II digested samples of *B. juncea*. *S. alba* contained a unique profile from all the species represented. Coupled to PCR data, the Southern blots allow for unambiguous identification of all genomes represented herein.

3.3.3. Gene Expression

The BN28 gene was originally cloned as a cDNA from acclimated leaf tissue of *B. napus* cv. Jet Neuf (Orr et al., 1992). BN28 hybridizes to a mRNA transcript of approximately 0.5 Kb in cold acclimated tissues of *B. napus*. Analysis of acclimated and nonacclimated Brassicas was accomplished by probing equally loaded blots of total RNA for the presence of BN28 transcript using radiolabelled BN28 cDNA. No message was detected in any of the nonacclimated samples. After 10 days of low temperature acclimation, all species showed high levels of expression of BN28 mRNA with the exception of *B. carinata* which showed a slightly lower level of accumulation (Fig 3.3). No significant differences in the size of the transcripts was detected in the different species examined.

Figure 3.1. Analysis of PCR Products. Gel electrophoresis of PCR products amplified using primers specific to the coding region of BN28: (Bn) *B. nigra*, (Bo) *B. oleracea*, (Br) *B. rapa*, *B. napus* cultivars (c) Cascade, (r) Rebel and (w) Westar, (Bc) *B. carinata*, (Bj) *B. juncea*, (ar) *S. arvensis* and (al) *S. alba*.

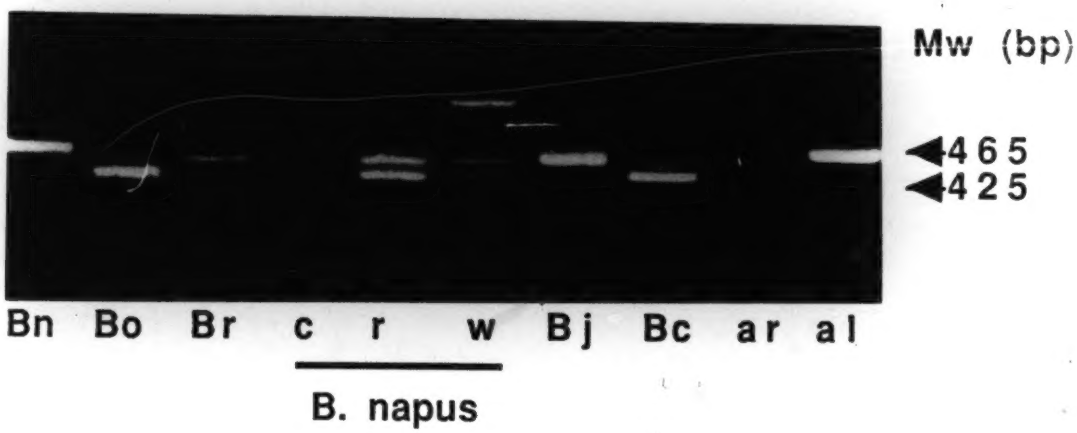
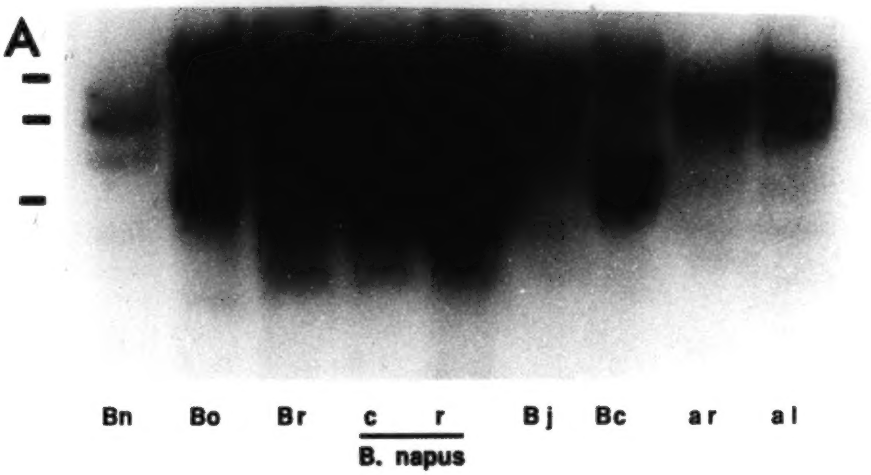


Figure 3.2. RFLP Analysis of BN28 Loci in the *Brassicaceae*. Southern blot analysis of the *Brassicaceae* using a BN28 cDNA probe against 20μg of DNA, **(A)** Digested with Eco RI. Band sizes are 2.8, 1.6 and 1.0 Kb respectively. **(B)** Digested with Pvu II. Band sizes are 2.5, 1.2 and 1.0 Kb respectively: Bn) *B. nigra*, (Bo) *B. oleracea*, (Br) *B. rapa*, *B. napus* cultivars (c) Cascade and (w) Westar, (Bc) *B. carinata*, (Bj) *B. juncea*, (ar)*S. arvensis* and (al) *S. alba*.

A



B

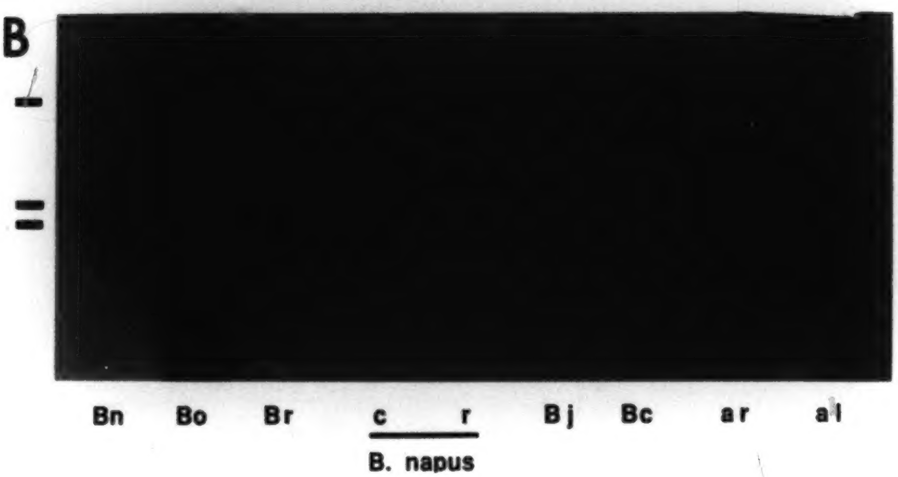
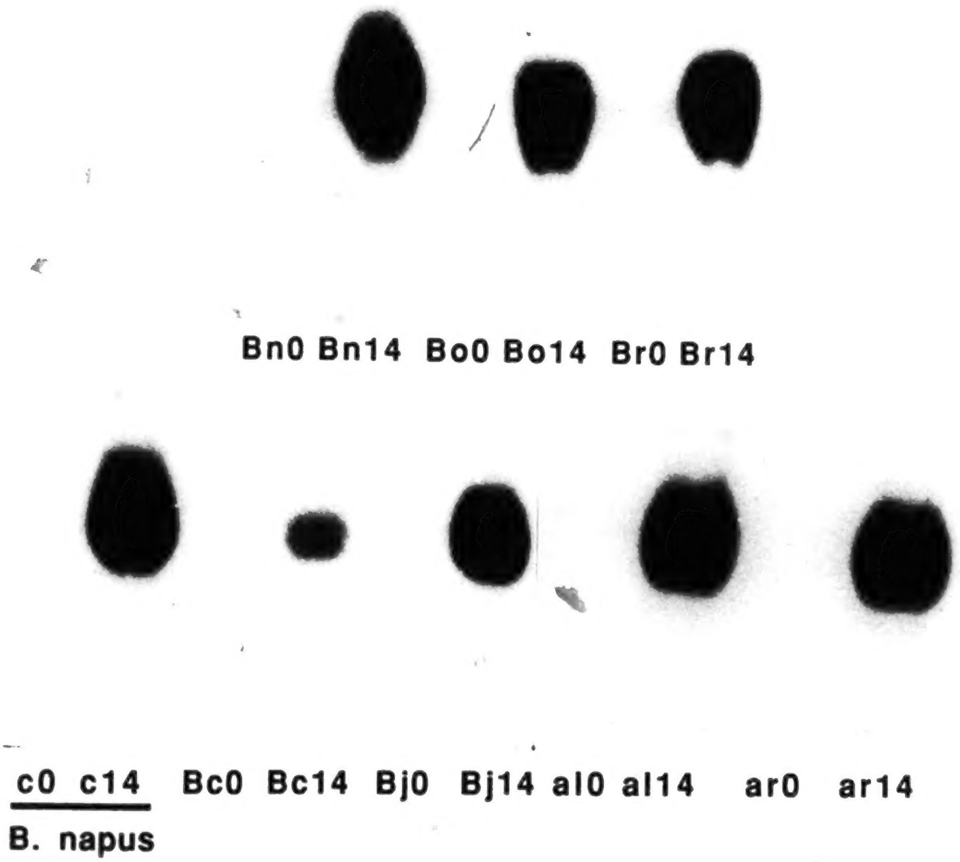


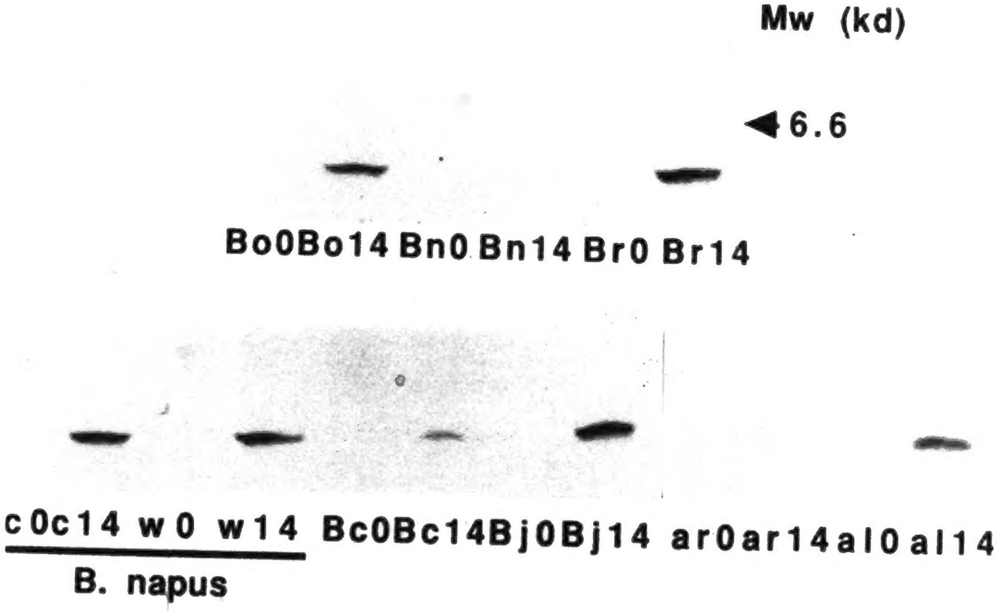
Figure 3.3. Northern Blot Analysis of Expression of BN28. 30µg total RNA from nonacclimated (**O days**) and low temperature acclimation (**14 days**) tissue was probed with cDNA probe BN28. BN28 probes to a fragment with a molecular weight of 0.5 Kb: (Bn) *B. nigra*, (Bo) *B. oleracea*, (Br) *B. rapa*, *B. napus* cultivar (c) Cascade, (Bc) *B. carinata*, (Bj) *B. juncea*, (ar) *S. arvensis* and (al) *S. alba*.


Bn0 Bn14 Bo0 Bo14 Br0 Br14
c0 c14 Bc0 Bc14 Bj0 Bj14 al0 al14 ar0 ar14
B. napus

3.3.4. Protein Expression

Accumulation of BN28 protein in nonacclimating and acclimating plants was evaluated by probing immunoblots with anti-BN28 polyclonal antibody (Boothe et al., 1995). The calculated molecular mass of BN28 protein is 6.6 kD, although the polypeptide migrates at a slightly lower apparent molecular mass. No accumulation of BN28 protein was detected in the nonacclimated samples. After 14 days of low temperature acclimation, all but two species examined showed protein accumulation (Fig 3.4). Again, there was no difference in apparent size or migration characteristics of BN28 protein in any of the species showing accumulation. There was no detectable signal in *B. nigra* and *S. arvensis.* Testing several samples at several developmental stages, including very young and mature leaves, or increasing the protein concentration to 200 µg failed to produce any detectable signal in these species (data not shown). It appears that although the gene structure may be different among species in the *Brassicaceae*, the translated BN28 protein maintains similar size and structure, except in *B nigra* and *S arvensis.*

Figure 3.4. Analysis of BN28 Protein Accumulation. Immunoblot of 30µg total protein from nonacclimated (**O days**). and acclimated (**14 days**) conditions using anti-Bn28 polyclonal serum: (Bn) *B. nigra*, (Bo) *B. oleracea*, (Br) *B. rapa*, *B. napus* cultivars (c) Cascade and (w) Westar, (Bc) *B. carinata*, (Bj) *B. juncea*, (ar) *S. arvensis* and (al) *S. alba*.


Mw (kd)
6.6
Bo0 Bo14 Bn0 Bn14 Br0 Br14
c0 c14 w0 w14 Bc0 Bc14 Bj0 Bj14 ar0 ar14 al0 al14
B. napus

3.4. DISCUSSION AND CONCLUSIONS

The manipulation of phenotypic traits using conventional breeding practices is a complex process. This is compounded in the *Brassicaceae* where several species are allotetraploids and may contain different copies of homologous genes.

In the past, breeding for improved resistance to low temperature stress has been difficult and consequently has met with very little success (Palta and Simmon, 1993). Regulation and structural analysis of many of the low temperature-induced genes has failed to provide any insight into function. We have recently characterized expression and accumulation of one of the low temperature induced genes BN28 (Boothe et al., 1995), originally isolated from *B. napus* cv. Jet Neuf (Orr et al., 1992), its spatial and temporal distribution (deBeus et al.,1997) and the biophysics of recombinant BN28 (Boothe et al., 1997). The purpose of the present study was to examine the genetic relationship of BN28. The scope of this study included the identification of possible homologues of BN28 in the three distinct genomes; AA, BB, CC, and the distribution of these homologues by each of the allotetraploids. Once the genetic relationship was established, examination of expression was completed in a select group of *Brassicaceae* species as represented by the work of U (1935).

The polymerase chain reaction (Saiki et al., 1988) has made it possible to amplify homologous sequences from both similar and diverse species. RFLP and PCR based markers have provided unprecedented insights into the evolution of nuclear genomes (Soltis and Soltis, 1993). It was possible to survey U's (1935) triangle with regard to the distribution of BN28, using primers specific for the 5' and 3' coding regions of the gene. Results reveal that each diploid parent contains a unique homologue of BN28 as indicated by differences in the size of the amplified fragments (Fig 3.1). *Brassica napus* (AACC) is the only allotetraploid to show the presence of BN28-like fragments from both diploid parents (*B. rapa* (AA) and *B. oleracea* (CC)). The other allotetraploids, *B. carinata* (AABB) and *B. juncea* (BBCC) appear to have inherited a single BN28 homologue from the (AA) and (CC) parent, respectively. Quiros et al. (1991) found a number of RAPD markers that failed to show any polymorphism between the three genomes represented in the *Brassicaceae*. This indicates a degree of conservation of homology within the three diploid genomes where previous work has uncovered genome-specific differences in isozymes, RFLPs and RAPDs within the *Brassicaceae* (Quiros et al., 1991; Quiros et al., 1987; Hosaka et al., 1990; Song et al., 1988). Our results suggest that BN28 is polymorphic in the diploid genomes. Results further indicate that at least some of the amphidiploid species tested did not inherit genes from both progenitor parent species or have undergone genome divergence so as to lose potential PCR priming sites.

Examination of gene structure using Southern blotting verified that unique

BN28 genes may be present in the diploid parents. The different restriction profiles were inherited by the allotetraploids (Fig 3.2) in a similar fashion as the results obtained using PCR (Fig 3.1) with the exception of *B. juncea*. *B. napus* carries profiles of both parents. *B. juncea* is similar to *B. rapa* and *B. carinata* is similar to *B. oleracea*. Interestingly, *S. arvensis* appears to have the same profile as *B. nigra*, while *S. alba* contains a unique profile to that of all the other Brassicas. This would suggest that specific homologues of BN28 are present in each of the diploid genomes and these homologues may have been transferred to individual allotetraploids, with *B. napus* being the only one to have acquired homologues from both original parents. We are unable to determine however, whether or not single genes or even groups of genes have been lost through species evolution or specifically transferred by original diploid progenitors giving rise to new allotetraploids. Because of the obligate outcrossing nature of the diploid *Brassicaceae*, more species may have to be examined before the exact number of BN28-like homologes in the *Brassicaceae* becomes apparent.

It is difficult to determine if sequence duplications reflect homeologous chromosomes of the diploid progenitor or similarities between the two progenitor genomes (Ferreira et al., 1994). Diploid Brassicas are considered to be secondary polyploids (Prakash and Hinata, 1980) derived by duplication of chromosomes from an extinct common ancestor having six chromosomes. Duplication and extensive redistribution then gave rise to the three diploids outlined by U (1935) (Richaria, 1937). Gene duplication in the allotetraploid Brassicas is not uncommon (Arus et al., 1991). Gene duplication has been reported in *B. rapa* and *B. oleracea* (Landry et al., 1992; Song et al., 1991; Kianian and Quiros, 1992). Results from the present study clearly shows homologous genes of BN28. Determining map positions of individual homologues may provide insight as to specific ancestry and degree of duplication that has occurred during the evolution of species.

Northern blot analysis confirmed that each species expresses a BN28-like gene that was induced in response to low temperature (Fig 3.3). Although different forms of BN28 were present, the coding regions appear to be homologous enough to hybridize to a common cDNA probe under high stringency conditions. Similar patterns of stress induction may suggest that each of the three homologues present in the *Brassicaceae* contain conserved regulatory regions.

Although all the *Brassicaceae* examined had low temperature-induced homologues of BN28, differences in protein expression were apparent (Fig 3.4). The lack of protein accumulation in *B. nigra* and *S. arvensis* suggests that gene silencing has occurred. Gene silencing has been found to occur in other allotetraploid species including *Pela rufa* (Gastony, 1988) and *Asplenium* (Werth et al., 1985). The nature of gene silencing is complex and includes a variety of different mutations (Soltis and Soltis, 1993). Such mutations can block transcription and/or translation, or the translation of a non-functional protein (Matzke and Matzke, 1995). In the case of *B. nigra* and *S. arvensis* it appears that translation of any antigenically similar protein did not occur. Given that transcripts are present, one possible explanation may be a frameshift mutation giving rise to a product that is not recognized by anti-BN28. Alternatively, nonsense mutations may have caused premature termination of any type of BN28-like protein.

Several of the documented genes that have been silenced are duplicates. Thus, it has been assumed that gene silencing occurs following a polyploid event (Grant, 1981). Both *B. nigra* and *S. arvensis* (SS) are diploid species and have undergone silencing of BN28. Several developmental stages were tested to ensure that no temporal regulation was occurring in the (BB) genome. In this case, silencing must lie within the sequence of the genes examined as they appear to be homologues of the (BB) genome. Similarly, silencing may be occurring in the (AABB) and (CCBB) allotetraploids. However, since there is no significant differences in the size of transcripts or protein from the different diploid progenitors, we are unable to differentiate between active and non-active genes in the allotetraploids surveyed.

In conclusion, the results from this study indicate all the Brassicas studied may contain a homologue of BN28 and express an RNA transcript when exposed to low temperature stress. However, gene silencing has occurred for BN28 in *B.*

nigra and *S. arvensis*. Present work regarding these genes involves study of gene structure, regulation of transcription and translation. Gene mapping studies will enable us to determine how many genes are present throughout the *Brassicaceae* and how they interact and function as specific homologues in a given species. Species-specific selection of alleles that produce protein immunologically similar to BN28 may then be best completed using sequence comparison and map position. *B.nigra* and *S. arvensis* will be used as models which do not produce detectable BN28 protein in expression studies designed to probe the function of BN28 protein. This should provide critical information needed to form an accurate model as to the function of BN28 during low temperature growth and acclimation.

3.5. LITERATURE CITED

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CHAPTER 4

Breaking the Linkage Between Freezing Tolerance and Vernalization in Winter *Brassica napus*

4.1. INTRODUCTION

Many Brassica cultivars are referred to as winter types because they are planted in the fall, overwinter and then complete development the following spring. During the overwintering period, the plants must meet a vernalization requirement to flower. This requirement is met by extended exposure to low, non-freezing temperatures. For example, *Brassica napus* cv. Cascade requires at least 6 weeks exposure to 4°C. Prior to vernalization, development is arrested at the rosette stage. Once the requirement is met, the plants bolt and become reproductive, first forming inflorescence meristems, followed shortly thereafter by flower meristems. Subsequent steps in floral development are then expected to be analogous in winter and spring types.

The vernalization requirement reduces the chance of premature flower induction following brief periods of warm weather during the winter months.

While this is advantageous to producers, it is to the detriment of breeders, who must add six weeks to every generation time for winter cultivars. As such, breeders are interested in gaining insight into the genetics of vernalization. Additionally, winter types have numerous attributes that could be beneficial if expressed in spring types. One approach to obtain these attributes in a spring type is to genetically remove the vernalization requirement in winter types. The resulting lines may then be assesed as a new source of variation in spring types.

The vernalization requirement has been studied in its role in accelerating flowering (Chintraruck and Ketellapper, 1969; Bagnall, 1988; Burn et al., 1993; Lee et al., 1993) and in relation to freezing tolerance (Fowler et al., 1995, 1996). There appears to be a broad range of responses from an absolute vernalization requirement, to a very weak requirement. Those with a weak requirement will eventually flower under favourable conditions in the absence of vernalization. Further, exposure to extended photoperiods and/or high light intensity may also overcome the low temperature requirement. This weak requirement is referred to as a quantitative response and is the response of most of the Arabidopsis ecotypes studied in this regard (Napp-Zinn, 1979; Lee et al., 1993). In these plants, exposure to vernalization conditions simply hastens floral induction. On the other hand, those with a strong vernalization requirement must be exposed to low temperature to induce floral development. This is referred to as a qualitative response, and is the response observed in winter Brassicas (Hodgson, 1978;

Tommey and Evans, 1991).

Little is known about the genetics of vernalization in Brassica. Early work on *B. napus* suggested that vernalization is controlled by two recessive genes (Thurling and Das, 1979). Similarly, studies on *B. oleracea* indicate polygenic inheritance (Pelofske and Baggett, 1979; Kennard and Osborn, 1994), with the annual growth habit being dominant (Baggett and Kean, 1989; Kennard and Osborn, 1994). More recently, linkage maps have shown one region that is strongly linked to vernalization requirement and days to flowering and two other regions with smaller effects on days to flowering both in *B. napus* (Ferreira et al., 1995) and *B. rapa* (Teutonico et al., 1994). These results suggest that vernalization requirment is controlled by vernalization specific loci and can be modified by flowering time loci.

The concept of a complex developmental pathway to explain floral induction in Arabidopsis (Martinez-Zapater and Somerville, 1990) is consistent with the presence of a number of interacting genes. Recent results from Arabidopsis further support this idea. A group of mutants, known as late flowering mutants (Korneef et al., 1991; Lee et al., 1993) have been identified, of which one, *co*, has a reduced response to vernalization. Lydiate and coworkers (Lagercrantz et al., 1996) recently comparatively mapped the *co* gene in Arabidopsis and *B. nigra*. Their results suggested that the homologue to *co* in *B. nigra* is an important QTL for flowering time in spring Brassica. As such, *co* is not specifically a vernalization gene (despite the fact that it was identified in a vernalization mutant) but rather, is a time to flowering gene.

To date, the only loci that appear to be true vernalization loci are *FRI* (Lee et al., 1993; Clarke and Dean, 1994; Lee and Amasino, 1995) and *FLC* (Lee et al., 1994). These are found on the vernalization dependent pathway (Dennis et al., 1996). Other loci, such as *vrn1* and *vrn2* (Chandler et al., 1996), *fca, fy* and *fve* (Burn et al., 1993) have arisen as recessive mutations of the spring ecotypes. These mutations are expected to confer a vernalization requirement by blocking the vernalization independent pathway (Martinez-Zapater and Somerville, 1990; Dennis et al., 1996).

The relationship between vernalization and freezing tolerance is unclear. Recent work on Arabidopsis suggests that freezing tolerance and vernalization are controlled by completely separate pathways (Chandler et al., 1996). It is difficult to draw a conclusion from the study, however, as the mutants were generated from the annual ecotype, Landsberg *erecta,* in which flowering is largely dictated by light conditions, with vernalization playing a secondary role. In addition, the ecotype only attains a moderate degree of freezing tolerance following exposure to low, non-freezing temperatures (Chandler et al., 1996).

In winter cereals, vernalization has been reported to be linked, either pleotropically or genetically, to freezing tolerance (Brule-Babel and Fowler, 1988). Recent work by Fowler and coworkers (1996) demonstrates an obligate relationship between vernalization and freezing tolerance, with tolerance decreasing significantly once the vernalization requirement is met. Galiba et al. (1995) have recently found recombination between specific vernalization and freezing tolerance loci in winter wheat. This is the first time that the two have ever been physically separated in any plant species.

There is limited evidence to suggest that freezing tolerance and vernalization are not linked in the Brassicas. Markowski and Rapacz (1994) demonstrated that there was no relationship between degree of freezing tolerance and the degree of vernalization required in winter *B. napus* lines. Genetic analysis by Osborn and coworkers showed that separate linkage groups in both *B. napus* and *B. rapa* contain genes for capacity to attain freezing tolerance and vernalization requirement (Ferreira et al., 1995; Teutonico et al., 1995).

Understanding the relationship between freezing tolerance and vernalization is further complicated by the genetics of freezing tolerance. Teutonico et al. (1995) showed that freezing tolerance in the Brassicas may be controlled by a number of genes grouped into linkage groups that are spread thoughout the genome. They also observed that regions linked to freezing tolerance in *B. rapa* were not linked with tolerance in *B. napus*. The complexity of the trait is compounded by the fact that plants have been shown to contain both inherent tolerance and acclimation-specific tolerance. Stone et al. (1993) in potatoto and Teutonico et al. (1993) in *B. rapa* showed that both species maintain independent levels of inherent and acclimation-specific freezing tolerance and both appear to be under separate genetic control (Stone et al., 1993).

Doubled haploid progeny of *B. napus* cv. Cascade and cv. Rebel crosses were developed to study the molecular genetics of freezing tolerance and vernalization. Cascade has an absolute vernalization response, while Rebel was bred and selected for a weak vernalization response. Rebel has little capacity for acclimation whereas Cascade is highly freezing tolerant. Rebel and Cascade were used as parent material for doubled haploid production. VERN- was isolated from a microspore-derived DH progeny of that cross and identified as a line that lacked a vernalization requirement, while retaining a very high degree of freezing tolerance. The present study characterizes the VERN- line with regard to plant development, freezing tolerance, and molecular genetics.

4.2. MATERIALS AND METHODS

4.2.1. Plant Material and Growth Conditions

Seed of *B. napus* cv. Cascade, cv. Rebel, F_1s, DH_1s, BC_1F_1 and BC_1F_2 were planted in pots containing a soil mixture of sand, peat and vermiculite (1:1:1) and grown under greenhouse conditions with a 16 hour photoperiod augmented with High Intensity Discharge (HID Sylvania Canada) sodium vapor lights to maintain a minimum light intensity of 275-300 µmol. m^{-2} s^{-1}. The temperature regime was 20°C day and 16°C night. VERN- plants were also grown, under greenhouse conditions with a non-inductive photoperiod of 10 hours. The temperature and light intensity were as above.

4.2.2. Development of Homozygous Lines

Reciprocal crosses between Cascade and Rebel were completed on 9 plants of each cultivar. Briefly, individual buds were opened using forceps sterilized in ethanol. Anthers, sepals and petals were removed prior to pollen dehiscence. Mature pollen from the appropriate male donor was used to pollinate the female stigma. The pollinated stigmas were covered with pollination bags until pod elongation was apparent and to ensure that no outcrossing could occur. Mature seed (F_1) was harvested, planted and grown as above. At the 4 leaf stage, plants were vernalized (see below) and returned to greenhouse conditions to be used as donor plants for microspore production. Microspores were released from the flower buds and cultured in liquid medium according to the methods of Coventry et al. (1988). At the late torpedo stage where cotyledons are apparent, embryos were transferred to solid medium (Lichter, 1982) for rooting. Rooted haploid plantlets were transferred to sterile soil and grown at 90% humidity until reaching the 4 leaf stage, at which time they were moved to greenhouse conditions, then vernalized accordingly. The haploid plants that had not doubled spontaneously were propagated by cuttings, colchicine treated and regrown according to Coventry et al. (1988). A single doubled (DH) plant was then used as the progenitor of each DH line. All fertile DH lines were covered with pollination bags for self-pollination, and homozygous seed was collected.

4.2.3. Vernalization and Acclimation

With the exception of the experiment designed to determine the minimum leaf number needed on VERN+ (a sib of VERN-) to flower, plants were moved, at the 4 leaf stage, to 4°C for 6 weeks. Minimum leaf number was determined by moving plants at the cotyledon stage and first, second, third and fourth leaf stage to 4°C for 3, 4, 5 and 6 weeks. The light intensity and photoperiod were maintained as above. Plants were then assessed for the presence of bolting and first anthesis. Limits of 95 days were set for the onset of bolting. Any plant that had not bolted was scored as vernalization requiring. Ninty-five days represents the approximate growth cycle for spring type cultivars.

4.2.4. Assessment of Freezing Tolerance

Freezing tolerance was completed on nonacclimated plants, plants that had been acclimated for 6 weeks at 4°C and deacclimated bolting plants, using an electrolyte leakage assay. The second and third leaves of 4 leaf stage plants and the youngest leaves of bolting plants were harvested and washed in distilled water. One-centimeter discs of leaf tissue were carefully removed, avoiding any veins, using a cork borer and placed on moistened filter paper (1 disc per filter) in small petri plates (2 filters per plate). Plates were placed in a programmable freezer and the temperature lowered to 0°C over a one hour period. A set of plates was removed at this temperature to act as the non-frozen control. The temperature was then lowered to -2.5°C over a one hour period and nucleation initiated by touching the filter paper with a metal probe cooled in liquid nitrogen. Samples were maintained overnight at -2.5°C. The temperature was then lowered at a constant rate of 2.5°C/hr and samples were removed at 2.5°C intervals between -2.5°C and -20°C. As samples were removed, they were allowed to thaw at 4°C for 12 hours. Electrolyte leakage was determined by placing the thawed discs (including paper) in tubes containing 10 mL of double distilled water and shaking overnight at room temperature. Freeze induced leakage was determined by measuring conductivity using a radiometer (Model CAM 83. Bach-Simpson LTD. London, Ontario, Canada.). Total leakage was determined after boiling samples for 3 minutes, cooling to room temperature, and shaking for at least 1 hour. Electrolyte leakage was expressed as a percentage of total for a given temperature and the TL_{50} was used as an indicator of lethal damage (Sukumaran and Weiser, 1972).

4.2.5. Phenotypic Characterization

Both vernalized and non-vernalized plants were grown under greenhouse conditions and assessed on the basis of the following parameters: days to fourth leaf; days to bolting; days to transition from vegetative to reproductive meristem; days to first flower; completion of flowering; maturity (measured by the presence of mature seed in the top ¾ of the primary raceme), number of expanded leaves; and yield assessment. The experiment was set up as three randomized replicates, having 15 plants per replicate for each parameter tested. Non-vernalized material was grown in the greenhouse for a minimum of 95 days to determine absolute vernalization requirements of the parents, F_1s and DH_1 lines as compared to the life cycle of a normal spring type. Field testing of non-vernalized plants was carried out as 3 replicates at 3 locations, 1 in Alberta and 2 in Saskatchewan. Seed was sown in 4 row nursery plots. Sampling was completed on 3 single plants and a bulk sample from each plot.

4.2.6. Segregation Analysis

VERN- was backcrossed to Cascade to produce BC_1F_1 plants, which were then self-pollinated to produce the BC_1F_2 generation. The vernalization requirement was determined as outlined above. BC_1F_2 were assessed for the appearance of bolting, and first anthesis as evidence for a lack of vernalization requirement. Absolute vernalization was gauged as presence versus absence of bolting after 95 days of growth.

Association of vernalization phenotype with any of the genotypic markers studied was completed using Fishers (2 tailed) test for association using SAS (version 6.3).

4.2.7. DNA Extraction

Leaf tissue was collected at the 4 leaf stage, flash frozen in liquid nitrogen and stored at -80°C to be used for genotypic assessment. DNA was extracted from approximately 5g of young leaf tissue by the method of Dellaporta (1983), with the following modifications: prior to isopropanol precipitation, samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged for 10 min at 10,000xg. Prior to ethanol precipitation, 5M NaCl was added to a final concentration of 2.5M (Fang et al., 1992). Samples were placed at -20°C for 30 min, pelleted at 4,500xg for 10 min at 4°C, rinsed 3 times in 70% ethanol and dried. Extracted DNA was resuspended in a minimal volume of TE8, (10mM Tris-HCl, pH8, 1mM EDTA) and quantified using ethidium bromide fluorometry (Karsten and Wallenberger, 1972). Briefly, as follows: 2µL of DNA was mixed with 2.4mL phosphate buffered saline (PBS) (170mM NaCl, 3.3mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 pH 7.2) and 0.1mL ethidium bromide (100 µg/mL). Samples were read on a Varian SF-330 spectrofluorometer with excitation at 360 nm and absorbance at 580 nm and were compared with to a calibration curve constructed using known quantities of lambda DNA.

4.2.8. Southern Blotting

Genomic DNA (20 µg) was restricted according to the manufacturer's instructions using Eco R1 or Hind III (Boeringer Mannheim). Digested samples were separated on an 0.8% agarose gel in 1X TAE at 80 volts for 3 hrs (Sambrook et al. 1989). Equal loading of restricted samples was assessed using ethidium bromide fluorescence of separated samples. Gels were capillary blotted onto Zeta-probe nylon membranes (Bio-Rad Laboratories, 3300 Regatta Blvd. Richmond, Ca 94804) using the semi-dry method of Rutledge et al. (1989). DNA was cross-linked to the membrane by illumination at 254 nm then baked at 80°C for 30 minutes under vacuum. Blots were prehybridized and hybridized according to the membrane manufacturer's instructions using 50% formamide at 43°C. Membranes were hybridized with a ^{32}PαdCTP(Amersham) labelled genomic probes (from either the cDNA or genomic libraries of Dr. Tom Osborn) (Random Priming Kit BRL #55567656) and rinsed at room temperature as follows: once for 2 min in 2X SSC and 0.1% SDS; once for 10 min in 1X SSC and 0.1% SDS; once for 10 min in 0.5X SSC and 0.1% SDS; and a final wash at 65°C for 10 min in 0.1X SSC and 0.1% SDS. Membranes were exposed to XAR-5 film with enhancing screens at -80°C for 2-5 days.

4.3. RESULTS

4.3.1. Phenotypic Characterization and Agronomic Performance

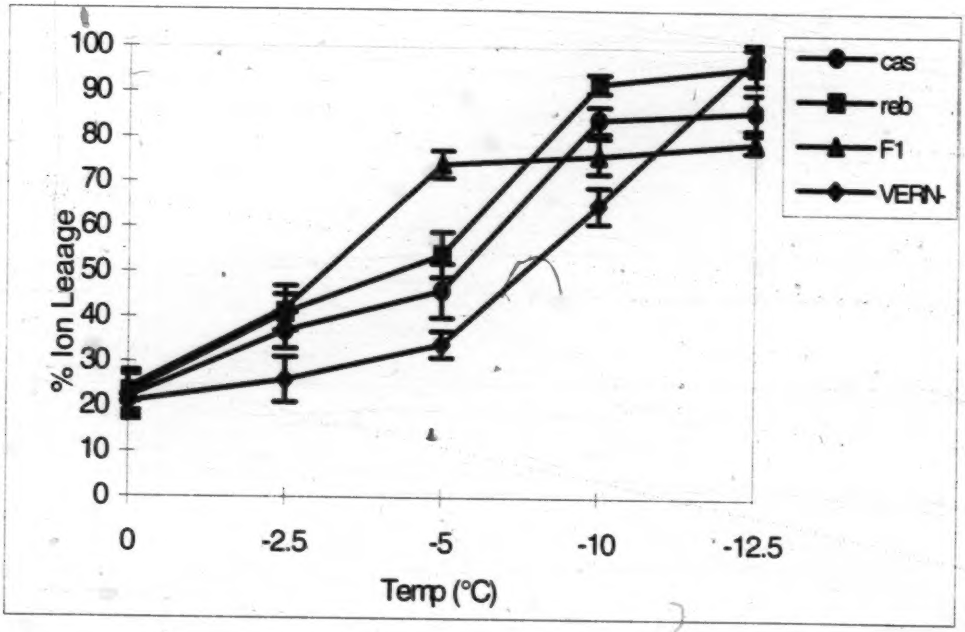
Phenotypic analysis of vernalized and non-vernalized DH lines revealed that one line did not require 6 weeks of vernalization to complete development and set viable seed . This line was called VERN- and is the focus of the present study. VERN- was compared to a sib line which requires vernalization and is termed VERN+

Although it was highly improbable that the photoperiod in the greenhouse could promote flowering in VERN-, this possibility had to be confirmed. Two approaches were taken: VERN- and the winter parents, Cascade and Rebel, were grown under a short (10 hr.) photoperiod The winter parents, were grown for over 180 days. The results indicated that the photoperiod was not inductive, as VERN- flowered under the short photoperiod (37 days), which is similar to normal growth conditions, while the winter parents failed to flower, even after an extended growth period. These results confirmed that VERN- had lost the vernalization requirement that was present in both parents.

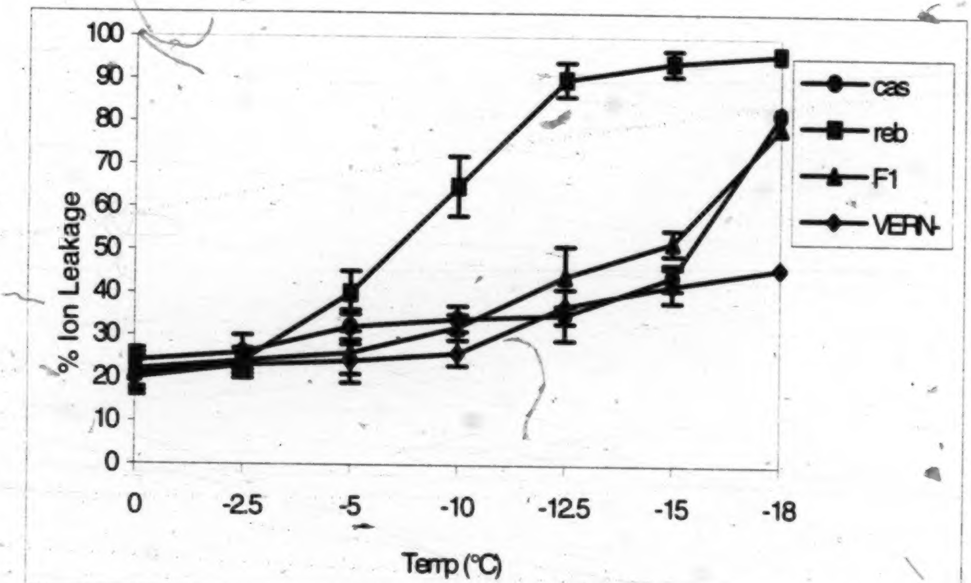
Following 6 weeks of acclimation, VERN- expressed a high degree of freezing tolerance (Fig. 4.1B) with a TL_{50} of $\geq$ -18°C, as compared to a TL_{50} of –15.5°C for Cascade and only –7.5°C for Rebel. In addition, inherent tolerance was high compared with the tolerant parent (TL_{50} of -8.5°C for VERN- and -5.5°C for Cascade) (Fig. 4.1A). This tolerance was expressed during both vegetative and reproductive development.

Figure 4.1. Inherent and Acclimation-Specific Freezing Tolerance in Cascade, Rebel, F$_1$ and DH$_1$ line VERN-. Assessment of ion leakage in nonacclimated and acclimated leaf tissue, +/- S.E. A) Inherent freezing tolerance. B) Acclimation-specific freezing tolerance.

A) Inherent Freezing Tolerance



B) Acclimation-Specific Freezing Tolerance



The loss of the vernalization requirement was confirmed in both greenhouse and field trials of original and self-pollinated seed (Table 4.1 and 4.2). In comparison with 3 commonly used spring checks; *B. napus* cvs. Altex, Legend and Alto, VERN- reached the 4 leaf stage and then bolted faster. In addition, VERN- was faster to reach the 4 leaf stage than either Cascade or Rebel. VERN- then continued to produce leaves rapidly, resulting in more leaves at flowering than were on the spring cultivars. Flowering in both VERN- and Legend began at 35 days and ceased at 50 days, 5 days earlier than either Altex or Alto. Further phenotypic characterization involved field assessments that compared VERN- with Excel and Quantum at 3 locations, 1 in Alberta (U of A farm, Edmonton) and 2 in Saskatchewan (Agriculture and Agrifood Canada research farm, Saskatoon)(Table 4.2). These cultivars were studied as blackleg tolerance is a required agronomic trait in the field, and Quantum is presently the best source of a resistant cultivar to this pathogen (Stringam et al., 1995). VERN- appeared to develop faster and have high yields in the preliminary trial. Excel, chosen for its earliness in the field, took 5 days longer than VERN- to reach the 4 leaf stage and was then 2 days later to initiate flowering and to complete maturation. Quantum was approximately 5 days slower in comparison to VERN- at all test times.

Table 4.1. Greenhouse Assessment of VERN-. Comparison of gross morphological characters associated with growth and development from germination through maturation. Data are the mean +/- S.E. (n=12) divided into 3 replicates, each having 4 randomized pots per line. Each pot contained 1 plant. Replicates were grown over a one year period.

Line	Fourth Leaf (days)	Bolting (days)	First Flower (days)	Last Flower (days)	Maturity (days)
VERN-	21	24	35	50	90
Rebel	23	n/a	n/a	n/a	n/a
Cascade	24	n/a	n/a	n/a	n/a
Altex	26	32	42	55	95
Legend	22	28	35	50	92
Alto	22	32	40	55	95

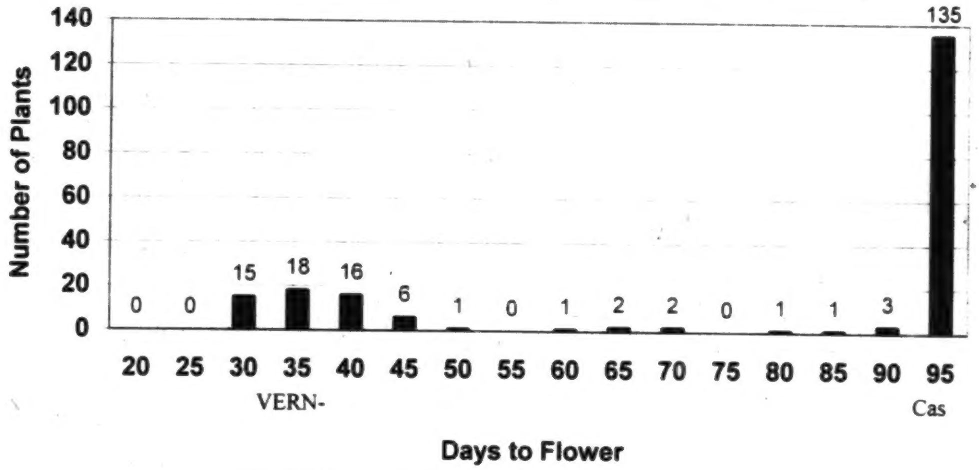
Table 4.2. Field Assessment of VERN-. Comparison of gross field characters of growth and development in VERN-, Excel and Quantum. Data were collected from 3 locations, 1 in Edmonton Alberta and 2 in Saskatoon Saskatchewan. Data were collectd from 5 plants in each plot (n=5).

Line	Fourth Leaf (days)	First Flower (days)	Maturity (days)
VERN-	21 +/- 0.75	46 +/- 1.2	102 +/- 1.6
Excel	26 +/- 0.60	48 +/- 1.4	104 +/- 2.0
Quantum	27 +/- 0.70	49 +/- 1.0	107 +/- 2.1

4.3.2. Distribution of VERN-

On the basis of the segregation ratio of BC_1F_1 and BC_1F_2, it appears that VERN- has a single homozygous recessive gene conferring the phenotype. Reciprocal backcrosses to Rebel and Cascade resulted in complementation of the VERN- phenotype and re-instatement of the vernalization requirement in 100% (40 of 40) BC_1F_1s tested. In the BC_1F_2, the phenotypic segregation ratio for 200 plants was 152 VERN+:48 VERN-; almost exactly a 3:1 ratio (Fig 4.2). This confirmed the homozygous, recessive nature of VERN-. The requirement for vernalization was assessed at 95 days for all the plants in this study. This represents the complete life cycle of a spring type. Therefore, if a line had not flowered after 95 days, it was scored as having an absolute requirement for vernalization.

Figure 4.2. Distribution of Vernalization. Distribution of vernalization in BC1F2 progeny from the reciprocal cross of Cascades with VERN-. (Includes 200 BC1F2 progeny).



4.3.3. Genomic Analysis

The genetic basis for VERN- was explored by probing Southern blots with genomic clones that had been shown to map to vernalization loci (Ferriera et al., 1995) (Fig. 4.3). Eco R1 digests of genomic DNA probed with WG6B10 show that VERN- is polymorphic to Cascade and Rebel. Interestingly, the same polymorphic difference was observed between Westar and the winter cultivars. The spring types both lack a band at 3.0 Kb and a band at 0.5 Kb. Although this suggests that there may be more than 1 loci responsible for the phenotype, the bands always appear to segregate with each other, indicating that they are closely linked. A different polymorphism was also noted between Cascade and Rebel at this locus. As anticipated from the phenotype of the BC_1F_1 generation, the vernalization requiring genotype was restored when VERN- was backcrossed to either Cascade or Rebel.

As phenotypic and genotypic complementation between the winter cultivars and VERN- had been demonstrated in the BC_1F_1 lines using the clone WG6B10, we were interested in determining the relative linkage. An example of the Southern blot is shown in Fig 4.4.

Figure 4.3. RFLP Complementation Analysis of Parents and F_1. A representative blot restricted with Eco R1 and probed with genomic clone WG6B10. Cascade (cas), Rebel (reb), and F_1 (F1s shown are reciprocals from Cas by VERN- cross).

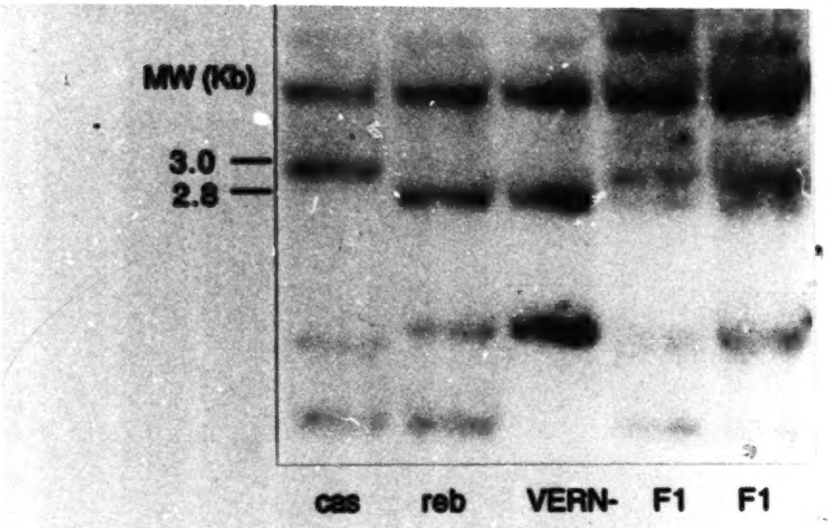
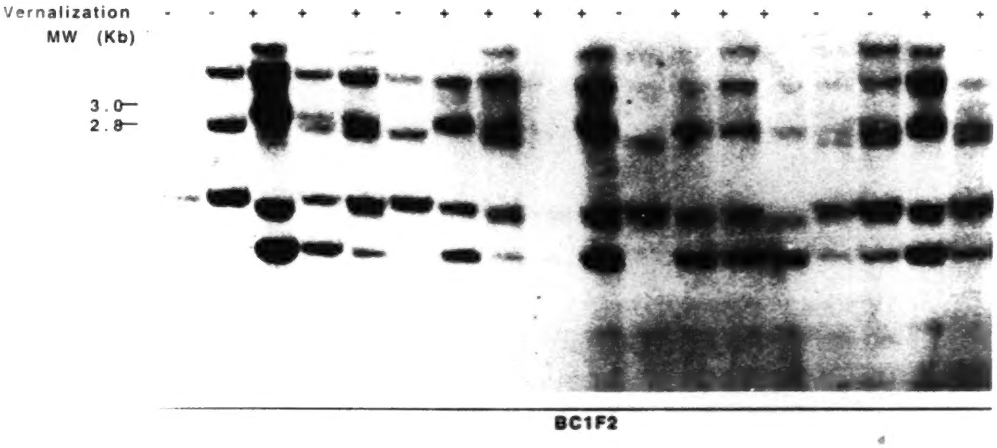

MW (Kb)
3.0
2.8
cas
reb
VERN-
F1
F1

Figure 4.4. RFLP Analysis of BC_1F_2 Segregants. A representative blot probed with genomic clone WG6B10. DNA was restricted with Eco R1. Vernalization requirement was denoted as: (+) requiring vernalization, and (-) not requiring vernalization.



Phenotypic analysis of 80 individual BC_1F_2 plants showed that 19 had the VERN- phenotype. The overall ratio was 61 VERN+:19 VERN-, indicating a possible recessive association in this population. Statistical analysis using Fishers 2-tailed test for association of genotype with phenotype showed a strong association for clone WG6B10 (< 0.005) (Table 4.3). In comparison, flanking clones WG7F3a and WG8G1 had association scores of .002 and .009, respectively, indicating that the VERN- phenotype displayed in this population may be more closely associated with WG6B10.

Table 4.3. Association Analysis of Backcross Progeny of Cascade x VERN- Cross. Probes from genomic clones (T.C. Osborn). Association between phenotype and scored genotype for each clone was calculated using Fishers (2-tailed) exact test. (0 indicates a strong association while 1 indicates no association) Phenotypic assessment of vernalization requirement was based on the presence of reproductive bud formation and was done at 35 and 70 days after planting.

Clone	Source	Polymorphic	size Kb	Association P value	Sample Size (n)
WG7F3a	B. napus	Yes	2.3	0.002	42
WG6B10	B. napus	Yes	3, 2.8	< 0.001	42
WG8G1b	B. napus	Yes	4.2	0.009	42

4.4. DISCUSSION AND CONCLUSIONS

The present study focused on developing and characterizing a spring type *Brassica napus* having a high degree of freezing tolerance. The germplasm was the result of a winter by winter cross of two *B. napus* cultivars, both of which have a vernalization requirement. It is not known whether the genotype that arose was the result of a genetic re-arrangement induced by colchicine or by tissue culture or by meiotic recombination of alleles in the parent cultivars that when in the correct combination remove the vernalization requirement. There is evidence, however, that significant genetic changes led to the loss of the vernalization requirement, as the polymorphism exhibited by genomic clone WG6B10 can be detected with a number of restriction enzymes in VERN-. (Fig.4.3 and 4.4). This suggests that we are dealing with a re-arrangment or deletion of more than a few base pairs.

The VERN- line differs significantly from other described vernalization mutants (Koorneef et al., 1991; Chandler et al., 1996; Martinez-Zapater et al., 1995), while having some similarities with the late flowering ecotypes of Arabidopsis (Lee et al., 1993; Clarke and Dean, 1994; Lee and Amasino, 1995; Martinez-Zapater and Somerville, 1990). First, it has a high degree of freezing tolerance (Fig. 4.1). Second, it appears to be on the vernalization dependent pathway as there does not appear to be any effects in this population from days to flowering genes. Finally, it is recessive for the spring growth habit. The latter two are characteristic of the late flowering ecotypes of Arabidopsis (Dennis et al., 1996; Lee et al., 1993; Clarke and Dean, 1994; Lee and Amasino, 1995).

Freezing tolerance in VERN- was greater than that of the tolerant parent, Cascade (Fig 4.3). This result is significant as a lack of adequate freezing tolerance in winter canola is always cited as a major constraint to expansion of the growing area. The improvement was noted in the DH lines, indicating that it may have occurred because of heterosis resulting from differences in the genomic nature of the parents. Teutonico et al. (1995) also noted transgressive segregants in their population. These results indicate there is potential to increase freezing tolerance in canola quality winter Brassica.

On one hand, freezing also severely limits the acreage seeded to spring canola. Spring frosts can kill or set back rosette-stage plants, resulting in yield reductions at the end of the growing season. On the other hand, fall frosts adversely affect quality (Johnson-Flanagan et al., 1991). Identification of VERN- indicates that these losses may be overcome. A spring type that has inherent tolerance of -8.5°C expressed at both the vegetative and reproductive stage will not be affected by frosts that rarely exceed -5°C.

VERN- appears to be very different from the vernalization mutants. Comparisons between VERN-, vernalized VERN+ and spring cultivars, demonstrated that the vernalization requirement is ameliorated in VERN-, as it flowered in the same time frame as the spring types. To my knowledge, all the vernalization mutations simply delay or accelerate flowering. In these mutants, changes in the vegetative phase is measured in terms of leaf number (Martinez-Zapater et al., 1995). VERN- also differs in this regard, as it produced more leaves in a shorter period of time than any of the other spring types examined in this study.

Earliness is a function of the length of both the vegetative phase and the reproductive phase. In VERN-, I noted that both phases were generally shorter relative to the spring cultivars. The fact that VERN- reached the 4 leaf stage in advance of the winter parents suggests that rapid vegetative growth is a function of a genetic alteration and is not a characteristic of the parents. Once flowering commenced, it was more prolific and proceeded more quickly in VERN-. Whether the short reproductive phase is a feature of winter types that has been retained in VERN-, or has arisen as a result of a genetic change remains to be seen. Studies of the Arabidopsis mutants provide little insight into this, as the length of the reproductive phase has not been the focus of study. One reference, Martinez-Zapater et al. (1995) indicates that a locus, FVE, may not only be involved in timing the transition from vegetative to reproductive, but may also play a role during all stages of plant development.

The agronomic traits measured in the present study demonstrate a number of features of VERN- that are important considerations for plant breeders. In addition, the results show that duration of vegetative growth is not a function of leaf number, nor is yield a function of the length of the reproductive phase as seen in VERN-. While it is appears that the VERN- locus may control the vernalization requirement, it is not known how it impacts upon leaf number, length of the reproductive program or number of flowers. VERN- shows potential as canola quality material, but until the effects of environment on the whole plant physiology and development are determined, the exact nature of this line will not be known.

To date, the genetic basis for the vernalization response has been studied predominantly in Arabidopsis mutants. Flowering in Arabidopsis mutants is hastened by exposure to one of high light intensity, extended photoperiod or low temperature. This is referred to as a quantitative response, as the plants will eventually flower in the absence of treatment (Lee et al., 1993). The genes responsible for the altered phenotype are considered to reside on the vernalization independent pathway (Dennis et al., 1996). In contrast, the VERN- line described in the present study flowers without any of the above mentioned treatments, whereas the parents will not flower unless vernalized. As such, the parents would be classified as having a qualitative response (Lee et al., 1993). This is similar to the late flowering ecotypes of Arabidopsis (Lee et al., 1993; Clarke and Dean; 1994; Lee and Amasino, 1995; Martinez-Zapater and Somerville, 1990). The genes that control this phenotype reside on the vernalization dependent pathway (Dennis et al., 1996).

Comparisons between the late flowering ecotypes of Arabidopsis and VERN- suggests that if VERN- resides on the vernalization dependent pathway, it is controlled by another, as yet uncharacterized locus. Analysis using RFLP has shown that VERN- does not appear to map with either FLC or FRI; FLC does not map to WG6B10 loci (Osborn, pers. comm), whereas VERN- does. It is also interesting to note that VERN-, which is recessive for the spring habit, maps near vernalization loci that are collectively dominant for the spring habit (Ferreira et al., 1995).

Little is known about the physiological relationship between freezing tolerance and vernalization. Chandler et al. (1996) suggested that freezing tolerance and vernalization may be perceived by different tissues and the signals then transduced separately. This was based on the retention of moderate freezing tolerance (-9°C) and the expression of low temperature-induced genes (*cor)* in the presence of a reduced vernalization requirement. Further evidence to support this exists in the literature. For example, the spring cultivar Westar achieves moderate freezing tolerance (-8°C), expresses the low temperature induced gene, BN28, and accumulates the gene product (Boothe et al., 1995). The parent of many of the late flowering ecotypes and mutants derived therein, Ler also exhibits a spring growth habit and attains moderate freezing tolerance. Thus, it is clear that the two responses are under independent control when in the spring growth habit.

The question remains, however, as to the physiological relationship between freezing tolerance and vernalization in the winter types. Results from the present study provide some insight into this. We have demonstrated a high degree of freezing tolerance in the absence of a vernalization requirement in VERN-, and both a high degree of freezing tolerance and a vernalization requirement in VERN+ and Cascade. Thus, it would appear that separate signals and pathways also exist for acclimation and vernalization in winter types.

The genetic relationship between freezing tolerance and vernalization has not been studied extensively in any winter crops except cereals. Research by Fowler et al. (1996) demonstrated that freezing tolerance and vernalization are inherited together and are, therefore, genetically linked. Galiba et al. (1995) have shown that the linkage can be broken at specific loci in winter wheat. The lack of linkage between these loci does not result in the loss of vernalization requirement. Research on the topic in Brassica has been restricted to spring by winter crosses (Teutonico et al., 1995) or correlative studies with a range of winter lines expressing different degrees of freezing tolerance and having variable vernalization requirements (Markowski and Rapacz, 1994).

Results from the present study clearly demonstrate that freezing tolerance and vernalization can be inherited separately. This would be expected on the basis of the genome mapping work by Osborn and his coworkers (Ferreira et al., 1995; Teutonico et al., 1995) who showed that separate linkage groups are apparent for both freezing tolerance and vernalization. Our results show that the VERN- line has lost the vernalization requirement while expressing a higher degree of freezing tolerance than is expressed by either parent, both in the absence of acclimation and following acclimation. As such, the linkage between vernalization and freezing tolerance has been broken in VERN-.

The findings from this study have implications that extend beyond furthering our understanding of how plants survive the winter and flower successfully in the spring: first, it indicates that there may be potential to access winter germplasm by simply removing the vernalization requirement in winter types, providing a new source of potential heterosis. Second, it provides homozygous germplasm that has a higher degree of acclimation-specific freezing tolerance than is currently available in winter canola; and finally, it provides homozygous germplasm that has superior inherent freezing tolerance than current spring canola cultivars.

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Chapter 5

Discussion and Conclusions

5.1. SUMMARY

One of the objectives of the study was to develop a plant system capable of addressing the complex nature of low temperature responses in *Brassica napus*. The result was 80 microspore-derived DH$_1$ lines displaying individual phenotypes for inherent freezing tolerance, acclimation-specific freezing tolerance and vernalization. The DH$_1$ line, VERN- is an example, where the need for vernalization has been removed, while high levels of both inherent freezing tolerance (-8.5°C) and acclimation-specific freezing tolerance (>-18°C) have been maintained. Other lines show that inherent freezing tolerance, acclimation-specific freezing tolerance and acclimation capacity are not correlated (See Appendix 6.1). Results from the present study shows that DH technology circumvents many of the problems associated with conventional breeding for improved freezing tolerance in canola. Containing a defined set of phenotypes, this DH population can now be analyzed in an attempt to gain a better understanding of low temperature responses in the *Brassicaceae*.

The source of parent material for this study was important. Two canola quality cultivars of *Brassica napus*, cv. Cascade and cv. Rebel were used. Cascade has an acclimated freezing tolerance of –15.5°C while Rebel can only acclimate to -7.5°C. Crosses completed in reciprocal fashion failed to reveal any effects of maternal cytoplasm (Appendix 6.1). The major genetic component of freezing tolerance in winter cultivars must, therefore, be within the nuclear component of the cell.

Breeding for improved tolerance to low temperature is difficult in plants and has met with very little success (Palta and Simon, 1993). Several reviews have provided reasons for the apparent lack of success, including complexity of control, and the narrow range of variability in the gene pool of many species (Blum, 1988). Work in this study has shown that increases in acclimation-specific freezing tolerance are possible, up to 18°C in some lines. The transgressive distribution exhibited indicates that optimal genes combinations exist for freezing tolerance. The continuous distribution confirms the quantitative nature of freezing tolerance in the Brassicas. Examination of the genetic components of individual DH lines may lead to defining important QTLs required for maximal expression of freezing tolerance. Concurrent with this work, Teutonico et al. (1995) using a winter by spring cross, reported inherent levels of freezing tolerance of –4.5°C and acclimated freezing tolerance levels of –13°C. In this system, the maximum values have been increased to –8.5°C for inherent freezing tolerance and greater than –18°C for acclimation-specific freezing tolerance. Winter by winter crosses, coupled to microspore technology, may lead to new winter cultivars of *Brassica napus* having higher levels of acclimation-specific freezing tolerance.

A number of low temperature-induced genes from *Brassica napus* and *Arabidopsis thaliana* were examined. It was found that they did not segregate with acclimation-specific freezing tolerance. This suggests that different post-transcriptional levels of control may be important in the expression of freezing tolerance. Alternatively, the results suggest that the effect of a single gene cannot be determined in a multi-gene system such as freezing tolerance. Individual phenotypes found in this DH population may provide needed insight into the role played by the low temperature-induced genes.

Analysis of inheritance of BN28 through out the *Brassicaceae* revealed that species specific loci exist. Multiple copies of genes in genera such as the *Brassicaceae* is not unexpected because of the common origin of homologous chromosomes. Not all of the allotetraploids appear to have inherited homologues from both progenitor parents. The implications of this are not presently known.

Freezing tolerance and vernalization have long been thought to be codependent as they are inherited together. With VERN-, I am the first person to unequivocally show that vernalization is not obligately inherited with freezing tolerance. Therefore, freezing tolerance and vernalization must be considered independent traits in *Brassica napus.*

Results reveal polymorphic differences between VERN- and VERN+ sibs using clone WG6B10, which has been previously mapped to a vernalization linkage group by Ferreira et al. (1995). This locus appears to segregate in a significant fashion with vernalization. At this time it is not clear whether the lack of the vernalization requirement in VERN- and its association with WG6B10 is on the primary linkage group causing the VERN- phenotype, or if it is a component of the phenotype. Continued mapping of the region surrounding WG6B10, as well as other areas of the genome, will clarify the genotype associated with the loss of the vernalization requirement in VERN-.

In the past, models have been developed in attempts to explain the requirement for vernalization and the induction of flowering in plants (Dennis et al., 1996; Haughn personal communication). The consensus is that two distinct pathways are involved, a vernalization dependent path and a vernalization independent path. The two pathways are thought to interact at several points. Much of the work to date has been completed using the numerous late flowering mutants of *Arabidopsis thaliana*. All these mutants lie on the vernalization independent pathway. Consequently, several loci have been identified on this pathway. On the other hand, only two genes have been placed on the vernalization dependent pathway. One reason for the poor progress is the lack of suitable material, as only late flowering ecotypes of *Arabidopsis* and winter cultivars appear to reside on the vernalization independent pathway. VERN- probably resides on the vernalization dependent pathway as well. As such, VERN- provides an opportunity to expand our knowledge of the vernalization dependent pathway.

Spring canola production in temperate climates is subject to yield losses attributed to late spring and early fall frosts (Johnson-Flanagan, 1989). Conventional spring cultivars have low levels of freezing tolerance (-2°C to -3°C). This may be improved with VERN-. Certainly VERN- shows good promise as a cultivar itself, as it is near canola quality (Appendix 6.3). It may also be used as a vehicle to breed increased inherent freezing tolerance (-8.5°C in VERN-) into spring cultivars. This will only be realized once DH lines are rated in a field situation, where the ultimate test for inherent and acclimation-specific freezing tolerance must be assessed.

5.2. FUTURE DIRECTIONS

The plant material developed in this present study provides the potential for future work in a number of areas. Some of the questions that come to mind are: "How are inherent and acclimation-specific freezing tolerance regulated?"; "How are the low temperature-induced genes involved in freezing tolerance?"; "What common principles are shared between vernalization and freezing tolerance?"; and "What is the function of vernalization and its affect on days to flowering in biennial cultivars?" Several experiments are currently being designed using the DH lines to answer a number of these questions. In depth screeing of a large population of species in the *Brassicaceae* may define exactly how many homologues of BN28 exist and how they are inherited. Fine scale mapping of VERN- will enable us to determine exactly what caused this line to exhibit a spring phenotype. Also, it creates the opportunity to eventually clone the VERN- gene. VERN- itself will have a significant impact on the understanding of vernalization in plants. Continuing mapping of freezing tolerance will enable the completion of QTL analysis in this population. Genomic subtractions between lines differing in inherent freezing tolerance and acclimation capacity will enable us to determine the genetic basis for each. Continued screening of freezing tolerance in this system will help to determine the gene combinations that give rise to optimal levels of freezing tolerance. Additional crosses to other winter types, coupled to microspore technology, may push the current levels of freezing tolerance even further.

In summary, use of the doubled haploid system has allowed for the genetic separation of inherent freezing tolerance, acclimation-specific freezing tolerance and vernalization in *Brassica napus*. It has provided phenotypic and genotypic evidence for the basis of these traits, and provided homozygous lines that can now be used for further study. In addition, this study has produced both spring and winter habit material that is canola quality and expresses high levels of inherent and acclimation-specific freezing tolerance. The use of this material in breeding programs is certain to impact on the future hardiness of the *Brassicaceae*.

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Appendix

6.1. Analysis of DH$_1$ Lines: Crossing, Doubling and Freezing Tolerance.

Line	Nonacclimated Freezing Tolerance (TL$_{50}$ °C)	Acclimation-Specific Freezing Tolerance (TL$_{50}$ °C)	Acclimation Capacity (TL$_{50}$ °C)	Doubling Action
1-401		-18		colchicine
1-402		-18		colchicine
1-403		-15		colchicine
1-404		-16		colchicine
1-100		-16		colchicine
1-101		-16		colchicine
1-102		-16		colchicine
3-101		-18		colchicine
3-102		-11		colchicine
3-103		-9		colchicine
3-104		-14		colchicine
3-105		-18		spontaneous
3-106		-11		colchicine
3-107		-18		colchicine
3-108		-15		colchicine
3-109		-12		colchicine
3-110		-14		colchicine
3-111		-18		colchicine
3-112		-11		colchicine
3-113	-4.2	-14	9.8	colchicine
3-114	-4.0	-11	7.0	colchicine
3-115	-5.8	-18	12.2	colchicine
3-116	-3.7	-18	14.3	colchicine
3-200		-18		colchicine
3-201		-9		colchicine
4-101		-16		colchicine
4-102	-4.4	-9	4.6	colchicine
4-103	-6.8	-9	1.2	colchicine
4-104		-8		colchicine
4-105	-2.3	-9	6.7	colchicine
4-106	-3.3	-11	5.7	colchicine
4-107	-4.8	-11	6.2	colchicine
4-108		-9		colchicine
4-109		-9		colchicine
6-100		-10		colchicine
6-200	-8.5	-18	9.9	colchicine
6-201	-3.9	-10	6.1	colchicine
6-202		-13		colchicine
6-203	-3.8	-12	12.2	colchicine
7-101	-2.9	-18	15.1	colchicine
8-100		-9		colchicine
8-101	-3.6	-9	6.4	colchicine

8-102	-3.4	-9	5.6	spontaneous
8-103		-9		colchicine
8-104		-8		colchicine
8-105		-18		colchicine
8-106		-4		colchicine
8-107	-3.3	-8	3.7	colchicine
8-108		-15		colchicine
8-109		-15		colchicine
8-110		-15		spontaneous
8-111		-10		colchicine
8-113		-17		colchicine
8-114		-18		colchicine
8-115		-15		colchicine
8-116		-14		colchicine
8-117		-9		colchicine
8-118		-8		colchicine
8-119		-16		colchicine
8-120		-13		colchicine
8-121		-17		colchicine
8-122	-6.3	-14	7.7	colchicine
8-123	-7.1	-18	10.9	colchicine
8-124	-2.2	-15	12.8	colchicine
8-125	-4.2	-18	13.8	colchicine
8-126	-5.4	-18	12.6	colchicine
8-127		-18		spontaneous
8-128		-16		colchicine
8-129		-12		colchicine
cas	-5.6	-16	9.9	n/a
reb	-4.4	-8	3.7	n/a
F1-1	-3.8	-16	10.7	n/a
F1-2		-16		n/a
F1-3		-10		n/a
F1-4		-8		n/a
F1-5		-9		n/a
F1-6		-14		n/a

TL$_{50}$s were calculated from ion leakage data collected on individual DH$_1$ lines (see Materials and Methods 2.2.5 for statistics and sample size).

Line number designates the plant material, i.e. x-yz. Even numbers (x) indicates that Rebel is the maternal parent. Odd number (x) indicates that Cascade is the maternal parent. y indicates plant number and z progeny number.

Acclimation capacity is the absolute difference between nonacclimated freezing tolerance and acclimated freezing tolerance.

Cascade, Rebel and F$_1$s are designated cas, reb and F1, respectively.

Appendix 6.2. Freezing Tolerance Linkage Groups in *Brassica rapa*. Relative position of linkage groups for acclimation-specific freezing tolerance (FTA) and acclimation capacity (FTB), as outlined by Teutonico et al. (1995).

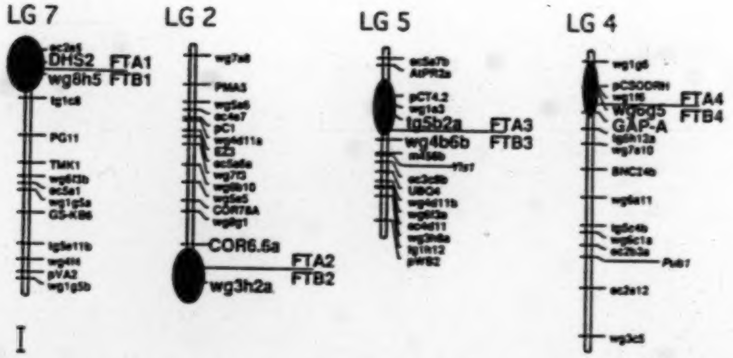

Fig. 3. QTL model for acclimated relative freezing (FTA) and acclimation ability (FTB) of *B. rapa* F3 families. Diameter of each ellipse is proportional to the percent variation explained by the individual QTL in the model and the length of the ellipse is proportional to the confidence interval for the QTL. Locus names are listed on the right of each LG. Linkage group designation and locus order from Teutonico and Osborn [44]. Loci flanking the likelihood peak of each QTL are indicated in large, bold type. The 20 cM linkage distance in Haldane map units is indicated.

From Teutonico et al. 1995.

Appendix 6.3. Quality Analysis of VERN-. Quality analysis of mature seed from VERN-. Collected from 3 locations (1 in Edmonton Alberta and 2 in Saskatoon Saskatchewan) from a single growing season. 2 single plants and a bulk sample were analyzed from each location. The data represent the mean for all completed analyses.

Sample	C12:0	C14:0	C16:0	C18:0	C20:0	C22:0	C24:0	C18:1	C22:1	Oil	Protein	Gluc
VERN-	0.02	0.03	3.4	1.8	0.66	0.35	0.35	67.2	0.02	47%	46%	12.2
Excel	0.03	0.04	3.6	0.21	1.7	0.66	0.05	65.5	0.11	45%	47%	11.4
*Cutlass	0	0	2.6	1.2	0.8	12.7	0.4	17.1	28.1	42%	n/a	n/a

Gluc= total glucosinolates

*Data from Saskatoon only

-Total Saturates (% found in total oil)

Total 6.3% (Standard is 6-9% of total oil)

-Total oleic acid (C18:1)

C18:1 = 67.2%

-Total erucic acid (C22:1)

C22:1 = 0.02% (must be < 2% of total oil)

-Total Oil (adjusted to 5% moisture on a whole seed basis)

47%

-Total Protein (adjusted to 5% moisture on a whole seed basis)

46% protein

-Total Glucosinolates (µmole/g oil-free meal)

12.2 µmole/g oil free meal.